

Altered Expression of Genes Encoding Cornulin and Repetin in Atopic Dermatitis

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Keywords

Atopic dermatitis · Cornulin · Gene expression · Genotype · Repetin

Abstract

Background: It is assumed that beside alterations in the filaggrin gene (*FLG*), disturbances within genes encoding other cornified envelope proteins are also involved in atopic dermatitis (AD). To identify new potential markers of AD, we studied the polymorphisms of genes encoding repetin (*RPTN*), cornulin (*CRNN*), and their expression in the skin of AD patients. **Methods:** Polymorphisms in *CRNN* (rs941934), *RPTN* (rs284544, rs28441202, rs3001978, and rs12117644), and *FLG* mutations (R2447X, S3247X) were analyzed by Taq-Man genotyping assay and by PCR-RFLP in the blood samples of 159 AD patients and 108 healthy subjects. The expression levels of *CRNN* and *RPTN* were determined by qRT-PCR in 34 AD skin samples (17 lesional and 17 nonlesional) and in 27 skin biopsies from healthy volunteers. The AD patients were recruited from the clinic of the university hospital between 2012 and 2014. **Results:** *CRNN* rs941934 (A allele) was associated with AD (OR 2.095, $p = 0.008$), a severe course of

disease ($p = 0.041$), elevated IgE levels ($p = 0.047$), eosinophilia ($p = 0.018$), and concomitant asthma ($p = 0.004$). The mRNA level of *CRNN* was decreased in the AD skin ($p = 0.041$). In the AD patients without *FLG* mutations, the CC genotype of *RPTN* rs3001978 was associated with AD (OR 0.39, $p = 0.037$), early age at onset ($p = 0.033$), pruritus ($p = 0.021$), severity of AD ($p = 0.045$), and concomitant asthma ($p = 0.041$). The elevated mRNA levels of *RPTN* in lesional ($p < 0.001$) and nonlesional ($p = 0.017$) AD skin were observed. **Conclusions:** The single-nucleotide polymorphisms of *CRNN* (rs941934) and *RPTN* (rs3001978, rs28441202) may contribute to AD development, but further studies on a larger group of AD patients are needed to verify this assumption.

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Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disease, with characteristic clinical pictures, concomitant pruritus, and diverse trigger factors [1]. AD affects adults and children with prevalence rates of 1–20% over the world, and is a socioeconomic challenge [2, 3]. The patho-

genesis is complicated and enforces a variety of therapeutic strategies and a customized individual approach for patients. The disease is the effect of genetic and environmental influences [4] as well as interactions between immunological factors and skin barrier defect [5, 6].

Skin barrier defect in AD concerns disorders in the stratum corneum (SC) involving lipid structure and functions, keratinocyte maturation, and formulation of the cornified envelope (CE) [1, 4–8]. CE proteins are encoded in the epidermal differentiation complex located on chromosome 1q21, and are divided into 4 families: late CE (LCE) proteins, small proline-rich proteins, EF-hand calcium-binding proteins of the S100A family, and S100-fused type proteins, i.e., profilaggrin (pro-FLG), hornerin, FLG-2, repetin (RPTN), cornulin (CRNN), and trichohyalin [9].

FLG maintains skin homeostasis. The products of FLG degradation are responsible for the formation of a natural moisturizing factor, retaining water in the SC and acidifying the SC. Thus, the lack of FLG that is frequently observed in AD leads to decreased hydration of the SC, increased transepidermal water loss, and the increased pH of the SC. The last activates serine proteases and increases epidermal barrier destruction. In consequence, the altered permeability of the epidermis allows allergens to penetrate it easily and provoke the inflammation process [5, 9].

FLG mutations are responsible for skin barrier defect in AD and are a known risk factor for AD development [10]. The most common *FLG* mutations in the European population are the 2282del4 and R501X mutations. However, *FLG* loss-of-function mutations are observed in 20% of AD subjects (and in up to 50% of severe AD subjects), but similar mutations are also observed in ichthyosis vulgaris, and in approximately 9% of the European population, without any concomitant inflammation [11, 12]. There are ethnic differences in the frequency of *FLG* mutations, and local differences exist even within Europeans regions [13, 14]. It is worth mentioning that the *FLG* deficiency in AD is caused not only by gene mutations but also by other exogenous and immunological factors which influence *FLG* expression [15, 16]. It has been proven that water, low humidity, skin irritants, sunburn, micro-organisms colonizing the skin, and the Th2 cytokines (e.g. IL-4, IL-13, IL-17, IL-22, IL-25, and IL-31) decrease the expression of *FLG*, independently of *FLG* mutations [1, 17]. However, impaired skin barrier function has also been shown in AD patients without *FLG* mutations [18]. Recently, the altered expression of genes encoding small proline-rich proteins and the LCE-like pro-

line-rich 1 protein (LELP1) in the skin of AD patients has been documented [19, 20].

All these facts support the suggestion that other AD risk factors exist in the epidermal differentiation complex apart from the prevalent *FLG* mutations [21–23]. An association between the single-nucleotide polymorphism (SNP), rs7534334, located 255 bp downstream of the *LELP1* gene, and the course of AD and atopic asthma has been reported [24, 25].

Another study points to CRNN [21]. CRNN is a 495-amino-acid protein, rich in glutamine (14.1%), with a calcium-binding motif similar to the S100 protein family [26]. It is located mainly in the granular layer of the epidermis. The function of CRNN is not clear, but it seems to be involved in CE formation, and is considered to be a marker of late epidermal differentiation [23, 27]. To date, only 1 report has provided data on the association of SNP rs941934 in *CRNN* with AD, and it suggested deepening the study on the possible involvement of the polymorphic forms of *CRNN* in AD development [23].

Besides CRNN, we also focused our study on RPTN. RPTN is a 784-amino-acid protein, rich in glutamine, with EF-hands of the S100 type and internal tandem repeats typical of CE protein precursors. It is localized in the stratum granulosum of the epidermis [28]. RPTN is not highly expressed in homeostatic epidermis, but can be upregulated upon alteration of the epidermal barrier [9, 28]. *RPTN* transcription is upregulated in *Klf4*-null mice (characterized by an impaired epidermal barrier function) [29] and *loricrin*-deficient mice [30].

These observations encouraged us to broaden our search for new AD risk factors among the SNPs of *CRNN* and *RPTN*. We believe that finding new risk factors among SNPs of CE proteins and gaining knowledge about abnormalities in protein levels in AD skin are crucial, especially in the context of a new strategy of AD treatment that is based on repair of the epidermal barrier, enhancing the transdermal delivery of FLG monomers to deficient skin, or the upregulation of *FLG* expression [7, 31].

The aim of our study was to search for the associations between SNPs of *CRNN* (rs941934) and *RPTN* (rs3001978, rs28441202, 12117644, rs 284544) with the risks and course of AD in the Polish population. We also analyzed a group of AD patients without *FLG* mutations. Moreover, we estimated the transcript level of *RPTN* and *CRNN* in lesional and nonlesional AD skin and in the skin of healthy subjects, and investigated the relation of these gene expression levels to clinical variables.

Table 1. List of primers, TaqMan probes and cycling conditions used for RT-PCR

Gene transcript	Primers	TaqMan probe
<i>CRNN</i> ¹ NM_016190.2	tggtcaaagatggatgcaag cctgtcctcccggtactgt	Universal ProbeLibrary Probe No. 51 (Roche)
<i>RPTN</i> ² NM_001122965.1	gctcttggctgagtttgag aggttcaagatggtttccaca	Universal ProbeLibrary Probe No. 65 (Roche)

Reverse transcription: at 48°C for 10 min and 95°C for 10 min. Amplification: at 95°C for 10 s and 60°C for 45 s for 40 cycles.

¹ Expression of this transcript was normalized to the transcript of *G6PD*.

² Expression of this transcript was normalized to the transcript of *TBP*.

Materials and Methods

Patients

The study population consisted of 267 subjects of Polish origin. The patients with AD were recruited between 2012 and 2014 from the outpatient clinics of Dermatology, Venereology and Allergology at the university hospital, on the basis of the diagnostic criteria of Hanifin and Rajka [32]. The patient group consisted of 159 patients aged 10–64 years. Patients receiving oral immunosuppressive treatment, other immunotherapies, or UV therapy during the last year were excluded from the study. The control group consisted of 108 healthy volunteers, ethnically matched on both gender and age, with no medical history of allergy, immunological diseases, or malignancies.

Skin Samples

For the examination of gene expression, 34 biopsies (2-mm punch biopsies of 3-mm depth) were taken from the AD patients (17 from lesional and 17 from nonlesional AD skin). The control group consisted of 27 healthy subjects recruited from the Department of Plastic Surgery. The size of the 2 groups subjected to skin biopsy was determined by the number of consents obtained.

The study was conducted with the approval (NKEBN/486/2011) of the local ethics committee (Independent Bioethics Commission for Research at the Medical University of Gdansk), and written consent was obtained from all the patients prior to enrolment.

Determination of IgE Levels

The total serum IgE levels were estimated by fluorescence enzyme immunoassay using the UniCAP 100 System (Phadia, Sweden) according to the manufacturer's instructions.

Determination of Atopic Disease Severity

The Severity Score of AD (SCORAD) helps to determine disease severity, i.e., mild AD: <25 points and severe AD: >50 points. A visual analog/numeric rating scale of 1–10 was employed to estimate the level of pruritus.

Genotyping

Genomic DNA was isolated using the MasterPure™ DNA purification kit for blood (Epicentre) in accordance with the attached

manual. The DNA concentration was determined by the fluorimetric method using the Qubit Quantitation Platform (Invitrogen). SNPs in the *RPTN* gene (rs28441202, rs3001978, and rs12117644) and mutations in the *FLG* gene (2282del4, R501X) were analyzed by the PCR-RFLP method. The PCR reaction (using specific primers designed for the analysis of individual polymorphisms) was performed with 500 ng of DNA, and the PCR products were digested by the appropriate restriction enzymes, i.e., rs28441202 by *HinfI*, rs3001978 by *AvrII*, rs12117644 by *SfcI*, R501X by *NlaIII*, and 2282del4 by *DraIII*. Polymorphisms in *CRNN* (rs941934) and *RPTN* (rs284544), and mutations in *FLG* (R2447X, S3247X) were analyzed by PCR-based TaqMan genotyping assay with a LightCycler 480 instrument (Roche, Mannheim, Germany).

Isolation of Total RNA from Skin Fragments

The skin fragments were homogenized in a sterile tube with 1 mL of TRI reagent (Sigma-Aldrich). Isolation of total RNA was carried out in accordance with the procedure used by Chomczynski and Sacchi [33]. RNA pellets were washed first with 96% and then 75% (v/v) ethanol, air-dried, and then resolved in diethyl pyrocarbonate-treated, thermosterilized water (50 µL), and stored at –20°C until further analysis.

Real-Time RT-PCR Analysis

The mRNA levels of *CRNN* and *RPTN* were analyzed by real-time RT-PCR with TaqMan primer-probe sets using the Path-ID™ Multiplex One-Step RT-PCR kit (Applied Biosystems). The reaction was performed in 96-well plates with a LightCycler 480 II instrument. The reference transcript (*TBP*) was used as an internal standard and was amplified together with each target gene transcript in the same well, using primers and probes as shown in Table 1. Thermocycling was conducted at a final volume of 10 µL of medium, containing: 1× Multiplex RT-PCR buffer, 1 µL of Multiplex enzyme mix, 0.5 µM of each PCR primer, 0.2 µM of each hybridization probe, and approximately 25 ng of total RNA isolated from the analyzed tissue (Table 1). The level of each analyzed transcript was normalized to that of the appropriate reference transcript. Analysis of the data was performed using the LightCycler 480 II software.

Table 2. Frequency of genotypes and alleles of *RPTN* and *CRNN* in AD patients and controls, with logistic regression analysis of the association of the *RPTN* and *CRNN* SNPs with AD, without excluding *FLG* mutations

Genotype and minor allele	AD patients (<i>n</i> = 159)	Healthy subjects (<i>n</i> = 108)	<i>p</i>	OR	95% CI	<i>p</i>
CRNN rs941934						
GG	112 (70.4)	88 (81.5)	0.019	0.542	0.542–0.980	0.043
GA	38 (23.9)	20 (18.5)		1.382	0.753–2.536	0.296
AA	9 (5.7)	0 (0)		13.698	0.789–237.885	0.072
G	262 (82.4)	196 (90.7)	0.007	0.477	0.277–0.828	0.008
A	56 (17.6)	20 (9.3)		2.095	1.217–3.606	0.008
RPTN rs28441202						
AA	156 (98.1)	106 (98.1)	0.989	0.981	0.161–5.972	0.984
AG	0 (0)	0 (0)		–	–	–
GG	3 (1.9)	2 (1.9)		1.019	0.167–6.204	0.985
A	312 (98.1)	212 (98.1)	0.97	0.981	0.274–3.519	0.977
G	6 (1.9)	4 (1.9)		1.019	0.284–3.655	0.977
RPTN rs3001978						
CC	8 (5)	13 (12)	0.091	0.387	0.155–0.969	0.043
CT	104 (65.4)	62 (57.4)		1.403	0.849–2.318	0.186
TT	47 (29.6)	33 (30.6)		0.954	0.560–1.625	0.862
C	120 (37.7)	88 (40.7)	0.48	0.882	0.619–1.256	0.485
T	198 (62.3)	128 (59.3)		1.134	0.797–1.616	0.485
RPTN rs12117644						
CC	0 (0)	0 (0)	0.503	–	–	–
CT	14 (13.2)	17 (16.3)		0.779	0.362–1.675	0.522
TT	92 (86.8)	87 (83.7)		1.284	0.597–2.761	0.522
C	14 (6.6)	17 (8.2)	0.530	0.794	0.381–1.656	0.539
T	189 (93.4)	191 (91.8)		1.259	0.604–2.625	0.539
Values express <i>n</i> (%). CI, confidence interval; OR, odds ratio.						

Values express n (%). CI, confidence interval; OR, odds ratio.

Statistical Analysis

The data from the investigation prepared specially for this study were statistically analyzed using Excel 2003 (Microsoft Corp., Redmond, WA, USA) and STATISTICA v8.0 (StatSoft, Tulsa, OK, USA). The Shapiro-Wilk test was used for checking normality. For the comparison of 2 groups, the Mann-Whitney U test was used. When multiple testing was required, the Kruskal-Wallis ANOVA with the Dunn post hoc test was performed. A logistic-regression model was used to calculate the odds ratio and 95% confidence intervals. Qualitative data were compared according to the number of cases in each category and/or their expected values by means of the Pearson χ^2 test, with the Yates correction or the Fisher exact test. The expression data were compared with the Mann-Whitney U test. For the comparison between lesional and nonlesional skin from the same patient, the Wilcoxon matched-pairs test was used. Associations between 2 variables were assessed based on the Spearman rank correlation coefficient (R). Estimation of linkage disequilibrium between pairs of SNPs was performed by employing the expectation maximization algorithm with the use of Arlequin v3.1 software [34]. $p < 0.05$ was regarded as statistically significant.

Results

Demographic Data: Genotyping

The patient group consisted of 159 subjects: 68 males (42.8%) and 91 females (57.2%). The male-to-female ratio was 0.7, and the mean age was 24.8 ± 12.3 years (range 10–64 years). The average SCORAD was 48.51 ± 22.09 (range 5–86). A mild course of AD was observed in 27 patients (17.8%), a moderate course in 72 patients (45.3%), and a severe course in 60 patients (37.7%). The mean age at disease onset was 4.8 ± 8.6 years. Early onset of AD (<2 years of age) was noted in 108 subjects (66%). Concomitant asthma existed in 44 AD patients (27.7%). The control group consisted of 108 subjects. The male-to-female ratio was 0.7, and the mean age was 27.1 ± 10.2 years (range 15–61).

Table 3. Relation of polymorphic variants of *CRNN* and *RPTN* with the clinical variables in all AD patients including those harboring *FLG* mutations ($n = 159$) and in a group of AD patients without *FLG* mutations ($n = 128$)

Genotype and minor allele	IgE serum level >100 IU/mL		Eosinophilia percentage		Concomitant asthma		SCORAD		Age at onset		Pruritus		Sex	
	-FLG	+FLG	-FLG	+FLG	-FLG	+FLG	-FLG	+FLG	-FLG	+FLG	-FLG	+FLG	-FLG	+FLG
<i>CRNN</i> rs941934														
GG														
GA	0.082	0.050	0.064	0.037	0.317	0.124	0.754	0.324	0.758	0.500	0.614	0.811	0.981	0.733
AA														
G														
A	0.061	0.047	0.079	0.018	0.065	0.004	0.051	0.041	0.722	0.986	0.825	0.432	0.539	0.411
<i>RPTN</i> rs28441202														
AA														
AG	0.824	0.738	0.772	0.586	0.438	0.297	0.522	0.488	0.067	0.049	0.175	0.129	0.729	0.664
GG														
A														
G	0.824	0.636	0.642	0.441	0.897	0.140	0.459	0.238	0.010	0.005	0.259	0.143	0.861	0.538
<i>RPTN</i> rs3001978														
CC														
CT	0.125	0.081	0.571	0.875	0.041	0.093	0.045	0.072	0.033	0.093	0.021	0.087	0.258	0.649
TT														
C														
T	0.259	0.117	0.108	0.893	0.341	0.159	0.092	0.070	0.972	0.785	0.090	0.072	0.176	0.983
<i>RPTN</i> rs12117644														
CC														
CT	0.305	0.178	0.527	0.358	0.671	0.441	0.218	0.973	0.529	0.301	0.523	0.471	0.281	0.993
TT														
C														
T	0.224	0.197	0.561	0.377	0.721	0.457	0.891	0.550	0.548	0.418	0.442	0.292	0.179	0.994
+FLG, harboring <i>FLG</i> mutations; -FLG, without <i>FLG</i> mutations.														

Demographic Data: Protein Expression

The group of patients consisted of 8 males (47.1%) and 9 females (52.9%). The male-to-female ratio was 0.88, and the mean age ($\pm$ SD) was 31.7 ± 12 years (range 21–64 years). The average SCORAD was 61.46 ± 14.06 (range 5–86), mean pruritus level 7.23 ± 1.76 , and mean IgE level $2,376 \pm 2,021$ kU/L. The mean age at disease onset was 7.6 ± 9.68 years. The control group consisted of 13 males and 14 females. The male-to-female ratio was 0.92, the mean age 39 ± 13.6 years (range 24–61 years), and mean IgE level 41.68 ± 42.5 kU/L.

Relation of *CRNN* Genotype with the Clinical Features of AD

The AA genotype of *CRNN* rs941934 was present only in the AD patients ($p = 0.019$), but was rare ($n = 9$; 5.7%).

The data presented in Table 2 indicate that the A allele was more frequent in the AD group than in the control group ($p = 0.007$). We found an association between the SNP of *CRNN* rs941934, an elevated IgE serum level ($p = 0.05$), and eosinophilia ($p = 0.037$). The GA genotype was more frequent in the group with elevated IgE than in the group with normal IgE ($p = 0.049$), and also in the group with eosinophilia compared to the group with a normal eosinophil count ($p = 0.03$). We observed that the A allele of *CRNN* rs941934 was associated with the severe course of AD ($p = 0.041$), an elevated IgE level ($p = 0.047$), eosinophilia ($p = 0.018$), and concomitant asthma ($p = 0.004$) (Table 3). Nevertheless, these associations were not sustained after excluding AD subjects with *FLG* mutations (2842del4, R501X, S3247X, and R2447X). Since the *CRNN* SNP, rs941934, is located approximately 100

kbp from the variants R501X and 2282del4 in *FLG*18, we examined whether rs941934 is in linkage disequilibrium (LD) with 2282del4. The LD analysis gave the value of $r^2 = 0$, which proved that there is no correlation between these 2 SNPs.

Expression Level of CRNN in AD Skin

The transcript level of *CRNN* in the AD skin was lower than in the healthy skin ($p = 0.027$). Furthermore, in the lesional skin of AD patients, the mRNA level of *CRNN* was significantly reduced compared to in the skin of healthy subjects (Fig. 1). There was no correlation of *CRNN* transcript level in AD skin and SCORAD ($p = 0.3$), pruritus ($p = 0.13$), or concomitant asthma ($p = 0.13$). We observed that the group of AD patients with an elevated IgE serum level or eosinophilia presented lower levels of *CRNN* mRNA than the controls, but without statistical significance ($p = 0.06$ and $p = 0.16$, respectively).

Relation of RPTN Genotype with Clinical Features of AD

There was no statistical significance of genotypes or allele frequency for *RPTN* rs28441202, rs3001978, and rs121176. As can be seen in Table 2, all the patients and healthy subjects were carriers of the AA genotype of *RPTN* rs284544.

Our analyses showed no associations between any genotypes or alleles of *RPTN* rs3001978 and sex, age, early onset of the disease, elevated IgE, eosinophilia, SCORAD, concomitant asthma, or pruritus (Table 3). However, after excluding carriers of *FLG* mutations from our analysis (2842del4, R501X, S3247X, and R2447X), the CC genotype of *RPTN* rs3001978 was associated with AD (OR = 0.39, $p = 0.037$) and a higher SCORAD ($p = 0.045$), early onset of the disease ($p = 0.033$), concomitant asthma ($p = 0.041$), and pruritus ($p = 0.021$) (Table 3). The patients with the CC genotype of *RPTN* rs3001978 suffered from more severe pruritus than those with the CT genotype ($p = 0.024$).

We observed an association between the *RPTN* SNP rs28441202 and early onset of the disease for genotype AA ($p = 0.049$) and for the A allele ($p = 0.005$). After excluding carriers of *FLG* mutations from our analysis (2842del4, R501X, S3247X, and R2447X), the A allele of *RPTN* rs28441202 was still associated with an early onset of AD ($p = 0.010$). There was no association between rs28441202 (A or G allele) and sex, age, elevated IgE, eosinophilia, SCORAD, concomitant asthma, or pruritus (Table 3).

There were also no associations between any genotypes or alleles of *RPTN* rs12117644 and sex, age, early onset of the disease, elevated IgE, eosinophilia, SCORAD, concomitant asthma, or pruritus (Table 3).

Expression Level of RPTN in AD Skin

The mRNA level of *RPTN* in AD skin was higher than in the controls ($p = 0.00002$). In lesional ($p = 0.000002$) and nonlesional ($p = 0.017$) AD skin, the mRNA levels of *RPTN* were elevated compared to the controls (Fig. 1). We did not observe any correlation between the mRNA level of *RPTN* and pruritus ($p = 0.76$), IgE level ($p = 0.1$), or SCORAD ($p = 0.43$). We have noted that the group of AD patients with elevated IgE or with a higher eosinophil count presented with a higher *RPTN* mRNA level, but this was without statistical significance ($p = 0.087$ and $p = 0.150$, respectively).

Discussion

The existence of some linkage to AD on chromosome 1q21 has been previously reported [35]. It has been suggested that AD risk factors might be located within the genes encoding CE proteins other than *FLG* [21–23].

This study has shown that some polymorphic variants of *CRNN* rs941934 and *RPTN* rs3001978 are associated with AD and the clinical characteristics of the disease. Our observations on the relation of polymorphic variants of *CRNN* rs941934 to AD development are comparable to those reported by Liedén et al. [23]. We observed an association between the rs941934 SNP and the level of IgE and eosinophilia. The A allele at rs941934 was associated with AD severity, elevated IgE, and concomitant asthma. However, these associations were not observed after excluding AD subjects with *FLG* mutations. This could be explained by the group of investigated subjects being too small, so further investigations are required.

Our observations on the reduced level of *CRNN* mRNA in lesional AD skin are consistent with a previous study [23] that reported a decreased mRNA level of *CRNN* in the lesional skin of AD patients, compared to nonlesional AD skin and skin from healthy controls. To resolve the question if the *CRNN* mRNA reduction in AD skin is caused by a gene polymorphism or whether other factors are involved, as is the case with *FLG*, whose expression is diminished by Th2 cytokines [1, 17], further studies are necessary. The reduction of *CRNN* mRNA and protein levels was previously reported in primary esophageal squamous cell carcinoma (SCC) and indicat-

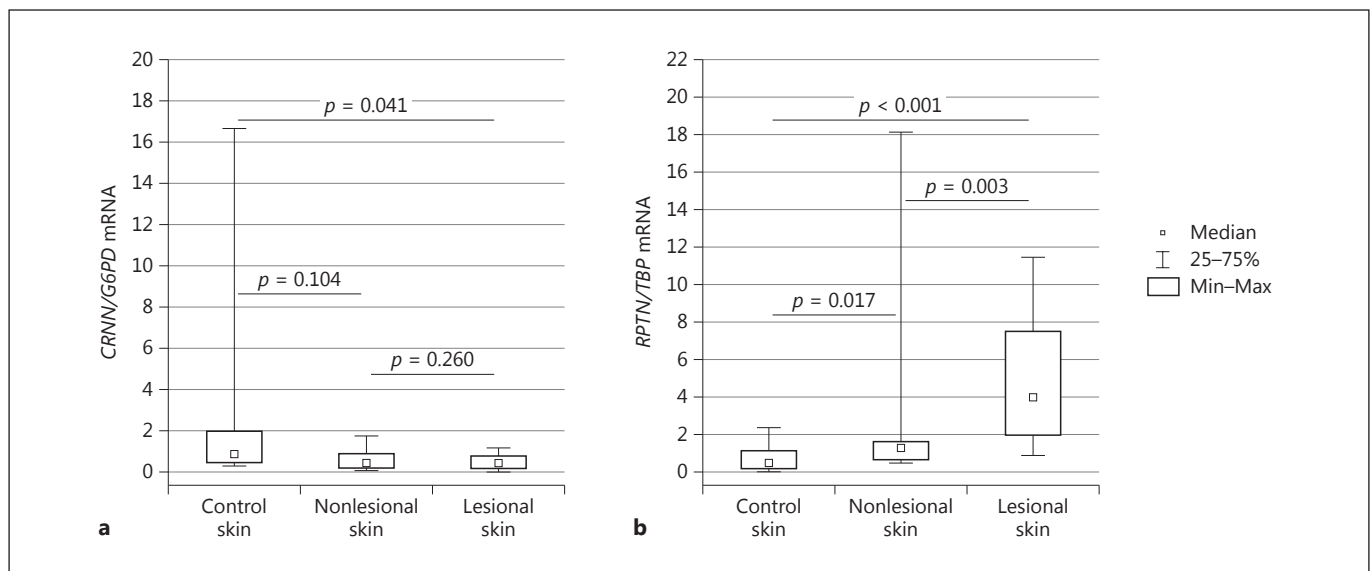


Fig. 1. The level of *CRNN* (a) and *RPTN* (b) transcripts in the skin of AD patients and of healthy subjects. The level of each mRNA normalized to that of the appropriate reference transcript (*G6PD* or *TBP*) was determined in the lesional ($n = 17$) and nonlesional ($n = 17$) skin of AD patients, and in the skin of healthy (control) subjects ($n = 27$). Min, minimum; Max, maximum.

ed as an independent prognostic factor for esophageal SCC [36]. Furthermore, in laryngeal SCC, the *CRNN* transcript is downregulated [37]. It has been demonstrated that *CRNN* is a tumor-suppressor gene [36]. On the other hand, there are studies reporting an increased risk of SCC among AD patients [38, 39]. It might be hypothesized that the increased risk of SCC in AD is associated with decreased *CRNN* mRNA levels observed in AD patients. However, again, this assumption needs further investigation.

The experiments on *Klf4*-null mice with impaired barrier function and on *loricrin*-deficient mice [29, 30] show the upregulation of *RPTN* transcription, which is consistent with our observations in humans. In our study, we found an increased *RPTN* mRNA level in the skin of AD patients whereas the *loricrin* mRNA level in both lesional and nonlesional AD skin was reduced (not shown). It has been postulated that an increase in the expression of *RPTN* is at least one of the compensatory mechanisms allowing for the organism to repair an altered epidermal barrier and compensate for the loss of the main epidermal CE component [30]. Our observations support such an assumption. *RPTN* contains high numbers of glutamine (approx. 20%), which might be the substrate for transglutaminases (TGMs) [30]. TGMs are the key enzymes that catalyze the cross-linking of epider-

mal proteins during the formation of the SC [40], and are capable of attaching and cross-linking lipids on the already cross-linked protein, TGM1 [41]. Levels of TGM mRNA and protein are increased in the skin from AD patients sensitized to *Malassezia sympodialis*, and are up-regulated under the condition of type stripping [40, 41]. Although TGM1 and TGM3 may be differently expressed in AD skin, the genetic analysis suggests that genetic variation in epidermal TGMs is not an important factor in AD susceptibility [41]. We observed that, in the group of AD patients without *FLG* mutations (2842del4, R501X, S3247X, and R2447X), the CC genotype of *RPTN* rs3001978 was associated with the severity of AD and itching. We also found that the AA genotype of *RPTN* rs28441202 and the A allele were associated with early onset of the disease and this was not related to the presence of *FLG* mutations.

It should be stressed that the major drawback of our study is the small number of AD cases and controls, especially when determining the gene expression levels from the skin biopsies. It should be emphasized, however, that our observations have shed a light on new SNPs that may be responsible for the course of AD. A confounding factor may be the lack of association of the polymorphic variants of *CRNN* rs941934 with AD in the group of patients without *FLG* 2282del14, especially when taking the LD analy-

sis into consideration, which indicated that the above 2 SNPs are not correlated to each other. This may also have been an effect of small group limitation.

To conclude, our study shows that the polymorphic variants of *CRNN* (rs941934) and *RPTN* (rs3001978 and rs28441202) may contribute to AD development, but further studies on a larger group of AD patients are needed to verify this assumption and the impact of the expression levels of these 2 genes. The more we know about skin barrier disorders, the better we can treat AD patients and prevent development of the atopic march.

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Disclosure Statement

No conflict of interest to declare.

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