

Original article

Cornulin, a marker of late epidermal differentiation, is down-regulated in eczema

Background: Eczema is a common chronic inflammatory skin disorder which shows strong genetic predisposition. To identify new potential molecular determinants of the disease pathogenesis, we performed a gene expression study in an eczema mouse model. This analysis identified a marked down regulation of the cornulin gene (*CRNN*), a member of the epidermal differentiation complex, in the eczema-like skin. We then investigated *CRNN* as an eczema candidate gene and studied its polymorphism and the expression in the skin of eczema patients.

Methods: An eczema-like phenotype was induced in mice by allergen (Der p2) patching. Gene expression analysis was performed with the subtractive suppression hybridization method and validated by real time PCR and the transmission disequilibrium test was used to test for genetic associations in 406 multiplex eczema families.

Results: Der p 2 patched mice developed a localized eczema and a Th 2 skewed systemic response. Real time PCR analysis confirmed a down regulation of *CRNN* mRNA in eczema-like skin in the mouse model and in human eczema. The *CRNN* polymorphism rs941934 was significantly associated with atopic eczema in the genetic analysis ($P = 0.006$), though only as part of an extended haplotype including a known associated variant (2282del4) in the filaggrin gene.

Conclusions: *CRNN* mRNA expression is decreased in eczematous skin. Further studies are needed to verify whether the associated cornulin polymorphism contribute to the genetic susceptibility in eczema.

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Eczema, previously referred to as atopic dermatitis (1), is a common chronic relapsing inflammatory skin disorder, which results from a complex interaction of genetic and environmental factors (2).

The skin forms an essential barrier against the external environment. This barrier structure resides within the outermost layers in the epidermis and is maintained by a balanced shedding of corneocytes (dead cells) from the

surface and a continual replacement from underneath by keratinocytes undergoing terminal differentiation (3, 4). Disturbances in the terminal differentiation and function of keratinocytes could impair the barrier function and thereby favor the penetration of microbes and allergens. A number of studies have shown that the barrier function in eczema is indeed altered (5). This can be seen in active skin lesions, where the inflammatory response plays a

role, but also in nonlesional areas, suggesting that barrier dysfunction is a central, underlying factor in eczema (6–13).

The identification of the genes that are dysregulated in the eczematous skin is thus likely to improve our understanding of eczema pathogenesis. A few studies have set up to address this issue in the past, by comparing the gene expression profiles of lesional and nonlesional skin in humans. While some studies have shown a higher expression of various chemokines, cytokines and other inflammatory mediators in lesional skin (14), a recent large scale microarray analysis instead pointed towards dysregulation of genes in the epidermal differentiation complex (EDC) as a disease characteristic (15). Notably, loss-of-function variants of the EDC member filaggrin (*FLG*) have also been recently associated with increased susceptibility to eczema (16).

The aim of this study was to identify new molecular determinants of eczema at the level of the skin. An eczema-like phenotype was induced in mice by epicutaneous patching with Der p 2, a major allergen from the house dust mite *Dermatophagoides pteronyssinus* (17, 18). By using the subtractive suppression hybridization (SSH) method and confirmatory real time PCR analysis, we detected the *CRNN* gene as differentially expressed in Der p 2 patched skin compared to control skin. The *CRNN* gene is located in the EDC, a known susceptibility region in eczema (19) on chromosome 1, and available functional data suggests that this protein might protect against apoptosis during cellular responses to stress (20).

We then studied the *CRNN* gene in human eczema. *CRNN* mRNA expression was compared in eczema patients and healthy controls and *CRNN* polymorphisms were tested for association by transmission disequilibrium test (TDT) analysis in a Swedish eczema family material.

Methods

Mouse studies

Sensitization protocol. Six to eight weeks old female BALB/c J mice, from The Jackson Laboratory (Bar Harbor, ME, USA), were shaved on the back and epicutaneously patched with 50 µg of recombinant Der p 2 (21) in 100 µl of PBS (or PBS alone as control) on a 1 cm² sterile gauze fixed with a hypoallergenic breathable dressing and support bandage from 3M (St Paul, MN, USA). Patches were applied for 4 days at week 1, 4 and 7, and the animals were sacrificed at day 50.

Skin histopathology. Skin biopsies from mice were collected day 50 and fixed in 10% buffered neutral formalin. Paraffin embedded biopsies were sectioned (4 µm) and hematoxylin and eosin stained.

Detection of Der p 2 specific antibodies. Specific IgG1, IgG2a and IgE were detected on Der p 2 coated 96-well plates with biotin-conjugated rat anti-mouse antibodies LO-MG1-2, LO-MG2a-7 and LO-ME-3, respectively from Serotec (Oxford, UK). Mouse recombinant IgG1 (107.3), IgG2a (G155-178), IgE (IgE-3) from BD Pharmingen (San Diego, CA, USA) were used as standards in

wells coated with a rat anti-mouse Ig light-chain antibody (R8-140, BD Pharmingen). ExtraAvidin®-alkaline phosphatase and *p*-nitro-phenyl phosphate substrate were from Sigma-Aldrich (St Louis, MO, USA). Data are expressed as ELISA units, corresponding to OD values with 1 ng/ml of standard at 405 nm.

Splenic T cell cultures and cytokine measurements. Spleens were collected on day 50 and homogenized in HBSS from Sigma-Aldrich. The cells were then treated with Red Blood Cell Lysis Buffer (Sigma-Aldrich), washed three times with HBSS, and resuspended in complete RPMI-1640 culture medium from Invitrogen-GIBCO (Carlsbad, CA, USA). Der p 2 (10 µg/ml) was added to the medium on day 1 of cell culture in 24-well plates, 4 × 10⁶ cells/well, for 3 days. Recombinant IL-2 was added at 10 U/ml, on day 3, 5 and 7. On day 10, live cells were harvested using the Ficoll-Paque Plus procedure from Amersham Biosciences AB (Uppsala, Sweden), washed three times with HBSS and seeded at 1 × 10⁵ cells/well in 96-well plates coated with anti-CD3 (145-2C11, NA/LE) and anti-CD28 (37.51, NA/LE) in the medium (both Ab from BD Pharmingen). Controls, without anti-CD3 and anti-CD28, were set up in parallel. Supernatants were collected at 48 h and cytokine production measured in an ELISA sandwich assay. Capturing and biotin-conjugated antibodies (mostly rat anti-mouse) were respectively R4-6A2 and XMG1.2 for IFN-γ, BVD4-1D11 and BVD6-24G2 for IL-4, TRFK5 and TRFK4 for IL-5, D8402E8 and D9302C12 for IL-9 from BD Pharmingen, and JES052A5 and goat anti mouse IL-10 for IL-10, and 38213 and goat anti-mouse IL-13 for IL-13 from R&D Systems (Minneapolis, MN, USA). Recombinant mouse cytokines were used as standards, IL-13 was from R&D systems and all others from BD Pharmingen.

Gene expression analysis. Skin biopsies were collected at day 50 from Der p 2 treated and PBS treated mice at the site of application, and total RNA extracted with Trizol Reagent (Invitrogen) according to manufacturer's instructions. Analysis of gene expression was performed by the SSH method (22) on the cDNA pools derived from 5 Der p 2 treated and 5 PBS treated skin biopsies. The resulting up and down-regulated sequences were cloned into bacteria and individual clones sequenced. BLAST and BLAT databank search tools were then used to identify the differentially expressed genes from sequence data. For real-time PCR experiments, 1 µg of total RNA from samples was reverse transcribed into cDNA with the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen) using an oligo(dT)_{12–18} in the reaction mixture. Primer Express™ 2.0 software from Applied Biosystems (Foster City, CA, USA) was used to design specific primers and real-time PCR reactions were carried out in triplicate for each gene in an ABI Prism 7500 SDS machine, using SYBR Green according to manufacturer's instructions (Applied Biosystems). After normalization with the endogenous housekeeping gene Hypoxanthine Phosphate Ribosyl Transferase (HPRT), relative gene expression levels were determined by the comparative C_T method. Sequences for mouse and human primers are available upon request from the corresponding author. All studies were approved by the local ethics committee.

Human studies

Study subjects. The genetic study material consisted of a previously described Swedish eczema family material (23). Families with at least two affected siblings above 4 years of age were included, for a total of 1514 individuals from 406 pedigrees. All siblings were diagnosed with eczema by the same dermatologist, based on clinical examination and according to the UK Working Party's diagnostic

criteria (24). Total serum IgE concentrations, and IgE specific for two mixtures of inhalant or food allergens were measured with the Phadia CAP Systems IgE FEIA, Phadiatop FEIA and RAST FEIA respectively (Phadia, Uppsala, Sweden), in all siblings. On the basis of specific IgE levels, the siblings were divided into two groups of atopic ($n = 588$) and nonatopic ($n = 333$) eczema patients, according to the revised nomenclature of the World Allergy Organization (1).

For the *CRNN* gene expression study, 15 paired skin biopsies from lesional and nonlesional areas, were collected from the back of eczema patients and from nine healthy individuals. The latter were all IgE negative (< 0.35 kU/l) for a panel of 11 common aero-allergens as determined by Phadiatop analysis (Phadia, Uppsala, Sweden), and had total IgE levels below 122 kU/l. In addition, 15 paired skin biopsies from patients with plaque psoriasis vulgaris were collected, lesional and nonlesional biopsies were taken from nearby areas, mainly on the knees and elbows. Age (mean age $\pm$ standard deviation) and gender distribution (females:males) in the different groups was as follows: eczema: 35 ± 11 years, 8:7; psoriasis: 42 ± 16 , 7:9; healthy controls: 37 ± 9 , 5:4. Real-time PCR analysis was carried out as previously described for the experiments in mice. All studies were approved by the local ethics committee and the subjects gave informed consent.

Genotyping. Six haplotype-tagging SNPs (rs10494275, rs4845766, rs941934, rs4240877, rs1923492 and rs1923493) were selected from the HapMap project (<http://www.hapmap.org>), covering the *CRNN* locus region on chromosome 1. Genotyping was performed on 1514 individuals by using allele-specific Taqman MGB probes labeled with fluorescent dyes FAM and VIC (TaqMan[®] SNP Genotyping Assays, Applied Biosystems), according to manufacturer's instructions. Allelic discrimination was performed with the ABI PRISM[®] 7900HT SDS and the SDS 2.2.1 program (Applied Biosystems).

Linkage disequilibrium (LD) between polymorphisms was calculated using the Haploview program (25).

Data and statistical analysis. Expression data were compared by Mann–Whitney test except for the comparison between lesional and nonlesional skin from the same patient, where the Wilcoxon matched pairs test was used. The TRANSMIT program was used for TDT analysis (26). Population Hardy–Weinberg equilibrium (HWE) was evaluated for each polymorphism using a chi-square test as implemented in the ZGENSTAT 1.128 software (H. Zazzi, unpublished data), with a cut-off value of $P > 0.001$. None of the analyzed polymorphisms deviated from HWE. *P*-values were not corrected for multiple comparisons.

Results

Induction of an eczema-like phenotype in mice

As shown by histological analysis in Fig. 1, Der p 2 treated mice presented with eczema-like features such as hyperplasia of the epidermal and dermal layer, hyperkeratosis and spongiosis and infiltration of inflammatory cells (mainly lymphocytes and eosinophils). This was not due to an irritant effect of Der p 2, as confirmed by a separate group of PBS treated mice, which were Der p 2 patched at the end of the protocol and showed normal histology (not shown). Der p 2 specific IgE, IgG1 and IgG2a progressively increased in allergen sensitized mice during the protocol period, while no increase was observed in control animals (Fig. 2A). As shown in

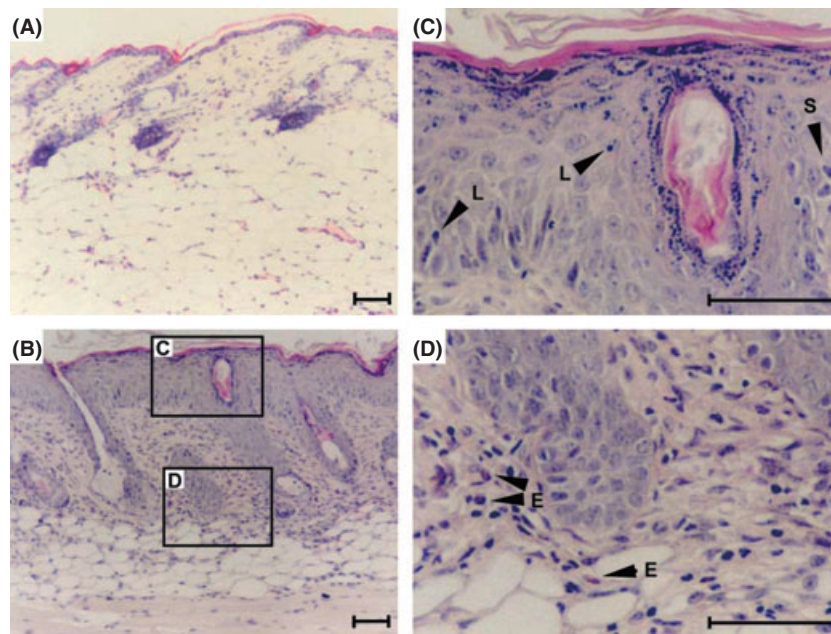


Figure 1. Skin histopathology induced by the Der p 2 allergen. Mice were repeatedly epicutaneously patched during a 50-day protocol with Der p 2 or PBS as control. Skin biopsies were collected (day 50), paraffin embedded, sectioned (4 μ m) and hematoxylin and eosin stained. Histology of PBS patched skin (A). Der p 2-patched skin showing hyperplasia of epidermal and dermal layers (B). Enlarged area from (B) showing lymphocyte (L) infiltrate in epidermis (C). hyperkeratosis, acanthosis and spongiosis (S) is also observed. Enlarged area from (B) showing eosinophils (E) and lymphocyte infiltrate in dermis (D). Bar represents 50 μ m.

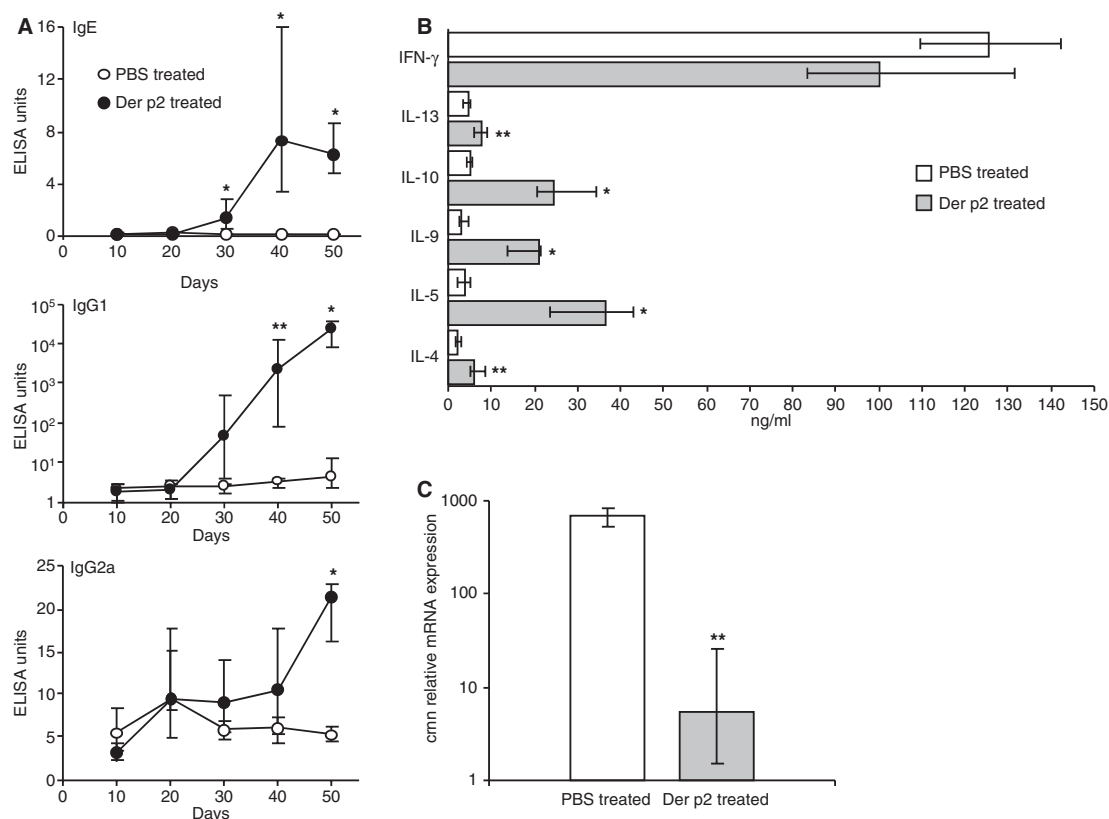


Figure 2. Der p 2 specific immunoglobulin in sera, cytokine production of splenic T cells and *crnn* mRNA expression in skin from mice patched with Der p 2 and PBS controls. Sera was collected throughout the protocol from Der p 2 ($n = 10$) and PBS control mice ($n = 10$) and levels of Der p 2 specific IgE, IgG1 and IgG2a was analyzed by ELISA (A). The Ab data is presented in ELISA units, one unit corresponding to the OD reading for 1 ng/ml of standard at 405 nm. IgG1 data is presented on a logarithmic scale. Cytokine production from splenocytes was analyzed by ELISA, cells from eight Der p 2 patched and eight PBS controls were analyzed (B). Expression of the *crnn* gene was analyzed by real time PCR in Der p 2 patched skin compared to PBS controls (C). $n = 6$ in both groups and the data presented on a logarithmic scale. All data are presented as median and the first and third quartile are shown by error bars. Statistical significance is indicated by (*) $P < 0.001$ and (**) $P < 0.01$.

Fig. 2B, splenic T cell cytokine profiles were also altered in allergen treated mice with significantly higher production of IL-4, IL-5, IL-9, IL-10 and IL-13 compared to control mice, while no difference was observed for IFN γ .

Identification of differentially expressed genes in the skin of Der p 2 patched mice compared to PBS controls. As a first step we used the SSH method to identify differentially expressed sequences in the mouse model. This analysis was performed on two pools of cDNA (Der p 2 treated vs PBS treated). BLAST and BLAT databank search tools were then used to identify 163 genes from the SSH clone libraries (up-/down-regulated). A complete list of genes is available upon request from the corresponding author. Additional *in silico* analysis was carried out on genes of unknown function for functional predictions (based on protein/domain homology searches with CD search, CDART, Prosite and pfam). From this data set we confirmed a number of genes by real time PCR but most striking was the down regulation of the *CRNN* gene in

Der p2 patched animals compared to controls, $P = 0.004$ (Fig. 2C).

Analysis of *CRNN* mRNA expression in the skin of eczema patients, psoriasis patients and healthy controls. The transcriptional level of *CRNN* was analyzed in skin biopsies from eczema and psoriasis patients (both non-lesional and lesional areas), and from healthy controls (Fig. 3). As previously observed in the mouse model, *CRNN* mRNA expression was reduced in the eczematous skin, with the majority of patients showing a 2-fold reduction or more in lesional areas compared to both nonlesional skin ($P = 0.009$) and skin from healthy controls ($P = 0.006$). Remarkably, reduced expression was not detected in psoriatic skin, either lesional or non-lesional, where *CRNN* mRNA levels were instead comparable to those detected in the skin of healthy controls.

Test for association between the *CRNN* gene and eczema. Six haplotype tagging single nucleotide poly-

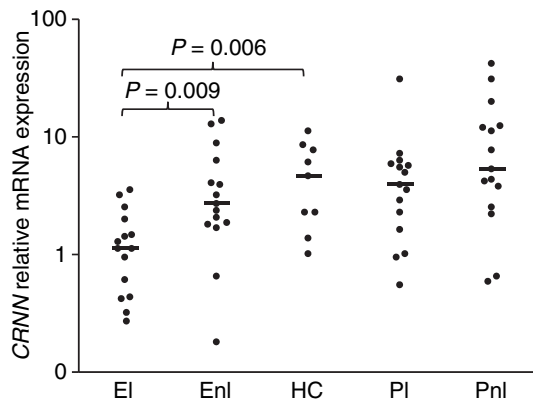


Figure 3. Analysis of *CRNN* mRNA expression in the skin of eczema and psoriasis patients and healthy controls by real time PCR. *CRNN* transcript levels in the skin of healthy controls (HC, $n = 9$) nonlesional area of eczema patients (EnI, $n = 15$) and psoriasis patients (PnI, $n = 15$) and lesional skin from the same patients (EI and PI, $n = 15$). Horizontal bars represent median values in each group and the data is presented on a logarithmic scale.

morphisms (SNP) in the gene region were genotyped in an eczema family material. As shown in Table 1, a TDT analysis with the TRANSMIT program revealed the SNP

rs941934 to be significantly associated with atopic eczema, and the minor allele to be over-transmitted in this group of patients ($P = 0.006$).

An analysis of LD in the region revealed the six SNPs to be contained within a single LD block, and to give rise to five haplotypes with a frequency over 2% (Table 2). One of these haplotypes, CCATAT, tagged by the SNP rs941934, was again associated with atopic eczema, and over-transmitted in this group of patients ($P = 0.004$).

The *CRNN* SNP rs941934 is located ~100 kilobases from the variants R510X and 2282del4 in the eczema gene *FLG* (16), and we have previously shown that these variants are associated with atopic eczema in our study material (27). This therefore prompted us to investigate whether the reported variants in the *FLG* gene are in LD with the rs941934 polymorphism. Analysis of LD showed $D' = 0.86$ and $R^2 = 0.26$ between rs941934 and the 2282del4 variant. Furthermore, extended haplotype analysis (Table 3), where *FLG* variants were included, revealed that the 2282del4 locus splits the associated *CRNN* haplotype into two different haplotypes, both tagged by the minor allele (A) at the rs941934 SNP. However, of these, only the haplotype carrying the risk 2282del4 variant was significantly associated with both eczema ($P = 0.002$) and atopic eczema ($P = 0.0002$) while the other haplotype showed no association

Table 1. Association of *CRNN* SNPs with eczema, atopic eczema and nonatopic eczema in a Swedish family material

SNP	Position*	MAF†	Eczema			Atopic eczema			Nonatopic eczema		
			n ‡	%§	P	n	%	P	N	%	P
rs10494275 (T)	2881922	0.068	128	51.8	0.52	88	54.6	0.20	38	45.5	0.36
rs4845766 (C)	2881637	0.366	646	50.4	0.64	410	50.8	0.51	227	49.4	0.70
rs941934 (A)	2880807	0.137	263	53.3	0.06	166	56.5	0.006	92	47.8	0.46
rs4240877 (T)	2877840	0.211	381	51.3	0.36	243	52.9	0.12	133	48.2	0.45
rs1923492 (A)	2875188	0.211	380	51.1	0.44	241	52.1	0.25	133	48.7	0.57
rs1923493 (G)	2874558	0.150	258	49.5	0.76	166	48.7	0.55	89	50.5	0.88

Minor allele within brackets.

*SNP position in contig NT_004487.18.

†Minor Allele Frequency (MAF) in the whole material.

‡Number of transmissions (n).

§Transmission frequency (%).

Table 2. Association of *CRNN* haplotypes with eczema, atopic eczema and Nonatopic eczema in a Swedish family material

Haplotypes							Eczema			Atopic eczema			Nonatopic eczema		
rs10494275	rs4845766	rs941934	rs4240877	rs1923492	rs1923493	HF*	n ‡	%‡	P	n	%	P	n	%	P
T	C	G	C	G	G	0.067	131	50.9	0.74	89	53.2	0.35	40	45.6	0.36
C	A	G	C	G	T	0.634	1144	49.8	0.65	712	49.3	0.35	422	50.7	0.43
C	C	G	T	A	T	0.076	130	47.9	0.43	84	47.2	0.41	45	49.1	0.85
C	C	A	T	A	T	0.137	266	53.6	0.04	170	56.7	0.004	91	48.1	0.51
C	C	G	C	G	G	0.086	146	47.2	0.25	88	44.3	0.07	56	52.4	0.56

*Haplotype frequency (HF) in the whole material.

‡Number of transmissions (n).

‡Transmission frequency (%).

Table 3. Genetic association of CRNN-FLG haplotypes with eczema, atopic eczema and non atopic eczema in a Swedish family material

Haplotypes									Eczema			Atopic eczema			Non atopic eczema		
rs10494275	rs4845766	rs941934	rs4240877	rs1923492	rs1923493	FLG_DEL4	FLG_501X	HF*	<i>n</i> †	%‡	<i>P</i>	<i>n</i>	%	<i>P</i>	<i>n</i>	%	<i>P</i>
T	C	G	C	G	G	WT	C	0.067	122	52.1	0.47	85	54.0	0.27	36	47.8	0.67
C	A	G	C	G	T	WT	C	0.606	980	49.1	0.17	623	48.4	0.06	350	50.6	0.58
C	A	G	C	G	T	WT	T	0.022	54	72.4	4.8×10^{-6}	40	76.8	1.1×10^{-5}	14	61.9	0.19
C	C	G	T	A	T	WT	C	0.077	121	48.7	0.65	79	47.9	0.56	41	50.1	0.99
C	C	A	T	A	T	WT§	C	0.086	132	47.5	0.34	75	48.3	0.62	54	45.5	0.27
C	C	A	T	A	T	Del§	C	0.046	96	61.3	0.002	74	66.6	0.0002	21	48.6	0.85
C	C	G	C	G	G	WT	C	0.084	129	46.2	0.15	79	43.4	0.05	47	51.5	0.75

*Haplotype frequency (HF) in the whole material.

†Number of transmissions (*n*).

‡Transmission frequency (%).

§Wild type abbreviated WT and deletion abbreviated Del for the *FLG* 2282del4 variant in haplotype presentation.

(Table 3). As expected, the extended haplotype tagged by the R501X (T) variant was also significantly associated with atopic eczema (Table 3).

Discussion

Through epicutaneous patching with a clinically relevant allergen (Der p 2 from the house dust mite), we induced a localized eczema in mice, which was associated with Der p 2 specific antibodies and increased production of Th2 cytokines from splenic T cells. These data are in accordance with a previously published study (21) and suggests that this mouse model represents a relevant experimental tool for the study of eczema. Gene expression analysis identified a striking reduction of *CRNN* mRNA levels in the skin of Der p2 treated mice compared to controls. Analysis of human samples confirmed a significant reduction of *CRNN* transcript levels in lesional skin from eczema patients, compared to both nonlesional skin and skin from healthy controls. Remarkably, this reduction appeared to be specific for eczema, since the same analysis performed on skin biopsies from patients suffering from psoriasis, another common skin disorder, did not disclose any significant difference compared to controls.

Given these findings, and the fact that the *CRNN* gene maps within the EDC on chromosome 1, where linkage to eczema has been reported (19), we also set out to determine whether genetic variation in the *CRNN* gene region could be associated with eczema susceptibility. Using a material of eczema families we identified one polymorphism, rs941934, upstream of the gene, which was significantly associated with atopic eczema. However, further analysis of LD between rs941934 and the recently identified variants in the eczema susceptibility gene *FLG* suggest that the association found in our material might be due to LD between rs941934 and the 2282del4 variant. The *CRNN* gene, also referred to as *C1orf10* or *SEP53*, codes for cornulin, a recently identified member of the

fused gene family (other members are trichohyalin, repetin, hornerin, *FLG*, and filaggrin 2), which combines functional calcium binding EF-hand domains at the *N*-terminus followed by tandem peptide repeats (28). *CRNN* is predominantly expressed in squamous epithelium with the highest levels found in esophagus and skin (20, 28–31). Contzler et al. (28) presented data suggesting that cornulin mRNA and protein expression in the epidermis is mainly localized to the granular layer. Calcium induced differentiation in cultured primary keratinocytes leads to an induction of cornulin expression, together with Keratin 10, thus indicating that cornulin likely represents a marker of late epidermal differentiation.

Cornulin function is still poorly understood, although some studies suggest that cornulin is up-regulated during, and might play a role in, stress response in squamous epithelium (20, 30, 32). Darragh et al. (20) showed that transfected cornulin can attenuate increases in intracellular free calcium and deoxycholic acid-induced apoptotic cell death in H1299 cells. Thus, although the mechanism by which cornulin may act as a survival factor still needs to be characterized, this protein might be involved in the protection from cell death and apoptosis. The results obtained in our analysis of cornulin expression in eczema and psoriasis patients may be interpreted to further support this hypothesis. In eczema, the skin is characterized by increased keratinocyte proliferation, impaired differentiation and increased apoptosis of keratinocytes (5, 10, 33, 34). The observed reduction of cornulin expression may thus be a part of the pathogenetic process leading to the epithelial barrier dysfunction that characterizes this disease. Indeed, the reduced 'protective' expression of other EDC members such as filaggrin, involucrin and loricrin is currently seen as a pathologic hallmark of the eczematous skin. Remarkably, it has been recently shown that Th2 cytokines, such as interleukin 4 and interleukin 13, can reduce the expression of involucrin and loricrin in

differentiating keratinocytes (35). Our observation of no reduction in cornulin expression in psoriasis may thus further reflect the fact that this disease is characterized by a Th1 cytokine profile and no increase in keratinocyte apoptosis. Altogether, these observations highlight the importance of the EDC members and their biological functions in the development of eczema.

In conclusion, we have identified *CRNN* as a new differentially expressed gene in eczema, and shown that genetic variation at the *CRNN* gene locus is associated with eczema susceptibility. Although the observed association between genetic variation at the *CRNN* locus and eczema are likely to be explained by LD with the 2282del4 variant in the *FLG* gene, the reduced levels of the *CRNN* transcript may be a part of an important mechanism in the disease development. Also, it is reasonable that other members of the EDC, in addition to or in interaction with *FLG*, influences eczema susceptibility (36). The study of

the expression and genetic variation of these genes is likely to provide important information in eczema pathogenesis.

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