Locus 1q21 Gene Expression Changes in Atopic Dermatitis Skin Lesions: Deregulation of Small Proline-Rich Region 1A

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Key Words

Epidermal differentiation complex • Atopic dermatitis • Cornified envelope • Gene expression • Real-time quantitative PCR

Abstract

Background: Discovery of the significant impact of filaggrin (*FLG*) mutations on the genetic predisposition to atopic dermatitis (AD) focused attention on the 1q21 locus, where not only *FLG* but also other epidermal genes are located. In the present study, we compared 1q21 gene expression in lesional versus nonlesional AD skin. **Methods:** A real-time quantitative PCR analysis of 10 1q21 genes, selected on the basis of a previous microarray study, was performed in skin biopsies from 33 individuals with AD. Three alternative pathway keratins were also evaluated. **Results:** In chronic AD skin lesions, we observed an increase in RNA encoding involucrin, S100 calcium-binding proteins A2 and A7–A9 and small proline-rich region (SPRR) proteins 1A and 2C, with fold changes ranging from 2.0 for *S100A2* to 15.4 for *S100A8* ($p < 0.001$, Bonferroni corrected), in parallel to the overexpression of the alternative pathway keratins 6A, 6B and 16. The loricrin (*LOR*) expression level was significantly decreased in lesional

AD skin (fold change 0.5; $p < 0.01$). The expression of the majority of 1q21 genes and alternative keratins was closely correlated; however, for *SPRR1A* (and *SPRR2C*) in lesional skin, the correlation with other genes was lost. **Conclusions:** We hypothesize that the deregulated increase in *SPRR1A* expression in chronic atopic skin lesions reflects an insufficient rise in *SPRR* transcripts, unable to compensate for the lack of *LOR* and thus contributing to the persistence of chronic AD skin lesions. Turning off the stress response in the skin may be regarded as a goal in the treatment of AD skin lesions, and *SPRR* genes might be targets for such an approach.

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Introduction

In recent years, much attention has been paid to the function of the epidermal skin barrier and to the molecular determinants of its deregulation in atopic dermatitis (AD). The cornified envelope (CE) is a key mechanism in barrier development, and disturbances in CE differentiation are central to the pathogenesis of AD skin lesions [1, 2]. In AD, the impaired function of the CE is genetically predetermined by loss-of-function mutations in the fil-

aggrin (*FLG*) gene, seen in approximately half of European patients [3, 4]. This gene is located on chromosome 1q21, within the human epidermal differentiation complex (EDC). At the protein level, FLG expression is very distinctly decreased or even obliterated in *FLG* mutation carriers [5], while mRNA levels may be less distinctly impaired [6]. Howell et al. [7] recently documented a decrease in *FLG* transcript and protein in acute AD lesions and related it to the influence of IL-4 and IL-13.

The EDC comprises several gene families [8], not only the family of fused genes encoding FLG itself and FLG-like proteins, among them FLG 2, repetin, trichohyalin, cornulin and hornerin. Short tandem peptide repeats in the central region characterize another gene family, which contains loricrin (*LOR*), involucrin (*IVL*) and a small proline-rich region (*SPRR*) gene cluster with not less than 11 genes [9, 10]. Another group is composed of *S100* family genes, with at least 14 transcripts encoding calcium-binding proteins with EF hand domains (11 of them are expressed in human epidermis). Also, 3 clusters of late envelope protein genes should be mentioned. During the physiological formation of the CE, several genes from each family are expressed in a coordinated way and the epidermal barrier activity is acquired in a stepwise manner [11], firstly with *IVL* expression, followed by the expression of *S100* proteins as well as *SPRR* proteins and, finally, *LOR* and *FLG* as late differentiation markers. A large number of CE proteins are transglutaminase substrates, used to assemble a cross-linked scaffold beneath the keratinocyte plasma membrane [12]. In normal epidermis, the major CE protein is *LOR*, which constitutes up to 70–80% of the total cross-linked protein mass and is cross-linked by *SPRR* proteins [1]. *FLG*, produced by post-translational proteolysis of the precursor protein, profilaggrin, plays a unique and pivotal role in cornification by promoting the formation of disulfide bonds and, as a result, aggregation of keratin filaments. Later on, *FLG* amino acid residues are modified and the molecule is degraded; this is instrumental in maintaining epidermal hydration and flexibility.

While the expression of *FLG* in AD has been studied extensively at both the RNA and protein level [5, 7], data concerning the expression of other CE genes are far less consistent [5, 13, 14]. We decided to study RNA expression because it has been proven that EDC genes are regulated mainly at the transcriptional level [15, 16]. Importantly, a large-scale DNA microarray analysis performed in AD skin biopsies by Sugiura et al. [6] indicated that several 1q21 locus genes responsible for the protective functions of the CE exhibited profound changes in their

transcript expression. The authors concluded that a key abnormality in AD might be deterioration of epidermal differentiation associated with altered expression of genes located on 1q21. However, the reported level of significance was not always sufficient and, even more essentially, no validation quantitative real-time reverse transcription PCR (QPCR) study has followed until now.

In our analysis, we evaluated the expression of the 1q21 genes identified by the microarray study mentioned above and compared it to that of the alternative keratinization pathway genes by applying a well-controlled QPCR approach in lesional and nonlesional skin of AD patients.

Methods

Patients

Thirty-three individuals (20 men and 13 women; age 18–45 years, median 24 years) with AD, diagnosed according to Hanifin and Rajka criteria, were included in the study after giving informed consent. The duration of AD ranged between 5 and 45 years. Atopic family history was positive in 45.5% of these patients (15/33), while personal history was positive in 85% (28/33). Standard skin prick tests (Allergopharma, Germany) were positive in all but 5 patients. Serum IgE (Allergopharma) was increased above 100 kU/l in 26 patients. The severity of AD was assessed by the SCORAD index [17]. No patients were treated with systemic corticosteroids or other immunosuppressive drugs. Systemic antihistamine drugs and/or local treatment were discontinued for at least 4 weeks before skin biopsy.

Full-thickness 4-mm punch skin biopsies (Stiefel Laboratories) of 3-mm depth were taken and immediately placed in RNA-later solution (Qiagen). Only chronic AD lesions were biopsied, as confirmed by histopathological assessment. The location of the skin biopsies was determined to avoid cosmetic consequences and to fulfill the condition of symmetry between lesional versus nonlesional areas, as well as keeping at least a 10-cm distance between nonlesional biopsy locations and other AD skin lesions. Arm skin was biopsied in 24 subjects, forearm skin or thigh skin in 3 and hand skin or trunk skin in 1. The study was approved by the local medical ethics committee and carried out according to Declaration of Helsinki regulations.

Isolation of RNA and cDNA Synthesis

Samples were stored at –80°C. Tissue was homogenized using a FastPrep FP120 instrument (lysing matrix D, Qbiogene). Total RNA was extracted using RNeasy Mini kits (Qiagen), including an on-column DNase digestion step, according to the manufacturer's instructions. RNA was quantified using a NanoDrop ND-1000 spectrophotometer, and sample quality was assessed by an Agilent 2100 Bioanalyzer using an RNA 6000 Nano Assay (Agilent Technologies). RNA quality, assessed by the RIN (RNA integrity) index, was within the 6.3–9.7 range. Paired samples from one individual did not exhibit significant differences in RNA quality (median difference in RIN between samples in pairs was 0.4). cDNA was synthesized from 200 ng of total RNA using an Om-

Table 1. Amplicons used for QPCR measurement of analyzed genes

Gene	Symbol	Gene ID	Primer sequence	Probe number and sequence ^a	Efficiency ^b	Gene function
Loricrin	LOR ^c	NM_000427	F 5'-CTCACCTTCCTGGTGCTT-3' R 5'-GAGGTCTTCACGCAGTCCA-3'	30 CCTCAGCC	1.91 95.6%	epidermal differentiation gene
Profilaggrin	FLG ^c	NM_002016	F 5'-CAGACACACAGTCAGTGTGAGC-3' R 5'-CAGACGTTTCCCCCTGACC-3'	21 CAGAGCCA	1.85 92.42%	epidermal differentiation gene
Involucrin	IVL ^c	NM_005547	F 5'-GGGCAGCTGGAGCTCTCT-3' R 5'-TCTGCTCCTCTGGGACCTC-3'	01 CCTGGAGC	1.88 94.1%	epidermal differentiation gene
Repetin	RPTN	NM_001122965	F 5'-GCTCTTGGCTGAGTTTGGAG-3' R 5'-GTTTCCACAGTCTCTGGGTCA-3'	65 TCCTCCAG	2 100%	epidermal differentiation gene
S100 calcium-binding protein A2	S100A2	NM_005978	F 5'-GGGAAATGAAGGAACCTTCTGC-3' R 5'-CCCCTCCTCATCCACTTTCT-3'	17 AGGAGCTG	1.75 87.74%	epidermal differentiation, immune function
S100 calcium-binding protein A7	S100A7	NM_002963	F 5'-CTGCTGACGATGATGAAGGA-3' R 5'-CGAGGTAATTTGTGCCCTTT-3'	60 CTTCCCCA	1.88 94%	epidermal differentiation, immune function
S100 calcium-binding protein A8	S100A8	NM_002964	F 5'-CAGCTGTCTTTCAGAAGACCTG-3' R 5'-GACGTCGATGATAGAGTTCAAGG-3'	78 AGCTGGAG	1.65 82.33%	epidermal differentiation, immune function
S100 calcium-binding protein A9	S100A9	NM_002965	F 5'-GTGCGAAAAGATCTGCAAAA-3' R 5'-TCAGCTGCTTGTCTGCATTT-3'	85 GACCTGGA	1.58 79.12%	epidermal differentiation, immune function
Small proline-rich region protein 1A	SPRR1A ^c	NM_005987	F 5'-CACCCCAAAGTGCCTGAG-3' R 5'-TTCTGCTTGGTCTTCTGCTG-3'	67 CTCCAGCA	1.63 81.72%	epidermal differentiation
Small proline-rich region protein 2C	SPRR2C	NM_006518	F 5'-TCTGCTTCGGAGAACCTGAT-3' R 5'-ACATGGCTCTGGACGCTTT-3'	10 CCACCTCC	2 100%	epidermal differentiation
Keratin 6A	KRT6A	NM_005554	F 5'-TTTGCCTCCTTCATCGACA-3' R 5'-GGGTCCACTTTGTTCCAGA-3'	01 CCTGGAGC	1.78 89.02%	alternative keratinization
Keratin 6B	KRT6B	NM_005555	F 5'-TTGCCTCCTTCATCGACAA-3' R 5'-TCTGCCTCACAGTCTTGGTG-3'	68 AGGAGCAG	1.66 83.25%	alternative keratinization
Keratin 16 (focal non-epidermolytic palmoplantar keratoderma)	KRT16	NM_005557	F 5'-ATCGAGGACCTGAGGAACAA-3' R 5'-GGCCAGTTCTGTCTCATAC-3'	64 CCAGGCTG	1.77 88.4%	alternative keratinization
Hydroxyacyl-coenzyme A dehydrogenase/3-ketoacyl-coenzyme A thiolase/enoyl-coenzyme A hydratase (trifunctional protein), alpha subunit	HADHA	NM_000182	F 5'-GTCTTGCGCCCATGATGT-3' R 5'-CAGCTTCTTCGGGTCAACTC-3'	65 TCCTCCAG	1.72 86.16%	reference house-keeping gene (control panel)
Eukaryotic translation initiation factor 5, transcript variant 1	EIF5	NM_001969	F 5'-TGGGTTTATGTCTTTATTTGACGA-3' R 5'-GCTATGTTTCCCCAATACAGGT-3'	46 GCAGCCAT	1.68 84.06%	reference house-keeping gene (control panel)
Eukaryotic translation initiation factor 3, subunit 10 theta, 150/170 kDa	EIF3S10	NM_003750	F 5'-AGTAGAGCGCCTGTACCATGA-3' R 5'-GCGTGTATTGGAGGCAGAAT-3'	61 TTGCCCAG	1.71 85.38%	reference house-keeping gene (control panel)
Ubiquitin-conjugating enzyme E2D2 (UBC4/5 homolog, yeast)	UBE2D2	NM_003339	F 5'-AATGGCAGCATTTGTCTTGA-3' R 5'-CACAAACAGAGAACAGATGGACAA-3'	67 CTCCAGCA	1.75 87.4%	reference house-keeping gene (control panel)
ATPase, H ⁺ transporting, lysosomal 31 kDa, V1 subunit E isoform 1	ATP6V1E	NM_001696	F 5'-AAGCCGGCTGGATCTCAT-3' R 5'-GCATTTGCACCAACAAGG-3'	03 CCCAGCAG	1.67 83.75%	reference house-keeping gene (control panel)

F = Forward; R = reverse.

^a From the Universal Probe Library, Roche.^b Calculated from the standard curve slope by a modified delta delta Ct method.^c Non-intron-spanning.

Fig. 1. Assessment of the reference genes used to normalize QPCR data. **a** Stability of the selected genes. **b** Pairwise differences between reference indexes based on different number of genes. Analysis was performed using geNorm software [20].

niscript cDNA synthesis kit (Qiagen), a mixture of oligo-dT primers (1 μ M) and random nonamer primers (4 μ M; Sigma) and 10 units of RNase inhibitor (Sigma) per reaction (37°C for 1 h).

Quantitative Real-Time Reverse Transcription PCR

Nine EDC genes selected by Sugiura et al. [6] in their gene expression profiling study were investigated; we also evaluated expression of the repetin gene. Three alternative pathway keratins, identified previously by the above-mentioned microarray study, were studied in parallel for the evaluation of the stress response of the epidermis. QPCR assay was performed using locked nucleic acid 5'-nuclease fluorescent probes (Universal Probe Library, Roche). Amplicons were designed using a Web-based application (www.roche-applied-science.com/sis/rtpcr/upl; for primer sequences, see table 1); intron-spanning amplicons were designed where possible (table 1). QPCR was carried out using an ABI Prism 7700 Sequence Detection System (Applied Biosystems) and a 96-well optical reaction plate. Five microliters of template cDNA (equivalent to 5 ng of total RNA) were added to 15 μ l of PCR reaction mix containing 10 μ l of TaqMan Universal PCR Master Mix (Applied Biosystems), 1 μ l of forward and reverse primers (200 nM), 1 μ l of probe (100 nM) and water. Thermal cycling conditions were as follows: 50°C for 2 min, then 95°C for 10 min followed by 40 cycles of PCR (95°C for 15 s and 60°C for 1 min). Each sample was examined in duplicate; the duplicates were assayed in independent runs. The mean coefficient of variability between duplicates was 3.03%; it ranged from 1.6 to 4.5% for different genes. A standard curve used throughout the experiments (serial dilutions of 200, 100, 40, 20, 8, 2, 0.4 and 0.04 ng, assayed in triplicate) was prepared from Human Reference RNA (Stratagene, used for reference genes) or normal skin RNA, mixed from 3 individual donors (used for the studied EDC genes). The efficiency for each gene was calculated from the standard curve slope by a modified delta delta Ct method (table 1), according to the formula given by Pfaffl [18, 19]; linearity of the obtained standard curves was verified by the R^2 coefficient. Gene expression values were obtained relative

to calibrator samples (the same as the samples used to prepare the standard curve, 200 ng) run in triplicate on each plate.

Gene Expression Normalization

To normalize the results obtained by QPCR, we analyzed the expression of 5 housekeeping genes (*HADHA*, *UBE2D2*, *EIF5*, *EIF3S10* and *ATP6V1E*). The chosen genes showed no differences in expression between lesional and nonlesional skin. To obtain the reference index (based on the geometric mean of the reference genes' expression values), geNorm software was used (fig. 1) [20]. All obtained results were normalized to this reference index, which ranged between 0.19 and 1.03. The most stable reference genes were *HADHA* and *EIF5*. The reference index obtained by combining more than 2 genes had increased stability.

Statistical Analysis

To determine between-group differences for paired samples, we used the Wilcoxon signed-rank nonparametric test, with exact computation of p values. p values were corrected for multiple comparisons by the Bonferroni method, using the number of genes tested. Results significant at a p value of 0.05 after Bonferroni correction were deemed significant. Correlations were assessed by the Pearson coefficient on $\log_{10}$ -transformed data; they were interpreted as significant at $p < 0.001$ (only results with $p < 0.05$ are shown). Hierarchical clustering was performed by average linkage and Pearson correlation as a distance metric. SPSS 13 (SPSS, Chicago, Ill., USA) was used for all statistical calculations.

Results

Expression of S100 Genes

S100A2, *S100A7*, *S100A8* and *S100A9* exhibited a strong and very significant upregulation in lesional AD skin, with fold changes ranging from 2.0 for *S100A2* to 15.4 for

Table 2. Gene expression comparison in nonlesional and lesional skin from individuals with AD

Gene	Normalized relative concentration			Difference between nonlesional and lesional skin ^a		Correlation between nonlesional and lesional skin ^c
	nonlesional skin (median)	lesional skin (median)	fold change in median values	p value	Bonferroni-corrected p value ^b	
Upregulated						
<i>S100A2</i>	3.07	5.98	2.0	0.00001	0.0001	n.s.
<i>S100A7</i>	22.5	172.7	7.7	<0.00001	0.00002	n.s.
<i>S100A8</i>	7.78	119.7	15.4	<0.00001	<0.00001	n.s.
<i>S100A9</i>	6.08	65.4	10.8	<0.00001	<0.00001	n.s.
<i>SPRR1A</i>	3.52	12.0	3.4	<0.00001	<0.00001	n.s.
<i>SPRR2C</i>	15.0	41.3	2.8	<0.00001	0.00005	n.s.
<i>IVL</i>	2.20	4.78	2.2	<0.00001	<0.00001	0.82
<i>Repetin</i>	1.48	1.79	1.2	n.s.	n.s.	n.s.
<i>Keratin 6A</i>	0.74	3.60	4.8	<0.00001	<0.00001	0.64
<i>Keratin 6B</i>	1.92	3.95	2.1	<0.00001	0.00004	n.s.
<i>Keratin 16</i>	0.35	4.7	13.5	<0.00001	<0.00001	n.s.
Downregulated						
<i>LOR</i>	0.78	0.39	0.5	0.0007	0.009	0.62
<i>FLG</i>	0.77	0.66	0.9	0.048	n.s	0.67

Both the differences between paired samples from lesional and nonlesional skin (Wilcoxon signed-rank test) and the correlations between lesional and nonlesional skin from the same individuals (Pearson correlation coefficient) were assessed. n.s. = Nonsignificant.

^a Wilcoxon signed-rank test (exact p values, 2-tailed).

^b Corrected for n = 13 comparisons.

^c Pearson correlation coefficients, calculated with log₁₀-transformed values, are shown for correlations significant at p < 0.001.

S100A8 (table 2). There was almost absolute correlation between *S100A8* and *S100A9* levels ($R = 0.95$ for nonlesional and 0.98 for lesional skin, $p < 0.001$; table 3). Close correlations were observed for *S100A7* and *S100A9* levels, and *S100A7* and *S100A8* levels, both in nonlesional and lesional skin, while the expression of *S100A2* exhibited a weak correlation with *S100A7* and *A9*, observed only in nonlesional skin.

Expression of *SPRR* Genes

Similarly to *S100* genes, the investigated *SPRR* transcripts were also distinctly increased in lesional AD skin, with an approximately 3-fold change (table 2). They also resembled *S100* genes in their lack of correlation between lesional and nonlesional skin levels (table 2). In nonlesional skin, the *SPRR* genes showed significant correlations with the *S100* gene expression levels, the strongest being for *SPRR2C/S100A7* ($R = 0.84$, $p < 0.001$; table 3). For *SPRR1A*, correlations with *LOR*, *FLG*, *SPRR2C* and *keratin 16*, significant at $p < 0.001$ in nonlesional skin, were absent in AD skin lesions, while 2 new correlations appeared, with *S100A2* and *IVL* (although the latter did

not fully meet our criterion of statistical significance at $p = 0.005$).

Expression of Other CE Genes: *LOR*, *IVL*, *FLG* and *Repetin*

IVL expression was distinctly upregulated in lesional AD skin (fold change 2.2 between lesional and nonlesional skin; table 2). In contrast, *LOR* expression showed downregulation, with a significant fold change of 0.5. Median *FLG* mRNA content was only slightly decreased in AD skin lesions, with an uncorrected p value of 0.048 (0.9-fold change between lesional and nonlesional skin), and appeared nonsignificant after Bonferroni correction for multiple comparisons. *Repetin* mRNA, which encoded an *FLG*-like peptide, also did not show any significant change.

The expression of *IVL*, *LOR* and *FLG* showed a significant correlation between nonlesional and lesional skin from the same individual ($p < 0.001$; table 2), the closest correlation being for *IVL* ($R = 0.82$). *FLG* RNA correlated with *LOR* in both AD lesions and intact skin (table 3), with similar correlation coefficients ($R = 0.83$

Table 3. Correlation between individual transcript levels in skin biopsies from patients with AD

	<i>S100A2</i>	<i>S100A7</i>	<i>S100A8</i>	<i>S100A9</i>	<i>SPRR1A</i>	<i>SPRR2C</i>	<i>LOR</i>	<i>FILAG</i>	<i>IVL</i>	<i>RPTN</i>	<i>KRT6A</i>	<i>KRT6B</i>	<i>KRT16</i>
<i>S100A2</i>	1				0.56 <0.001	0.47 0.01							
<i>S100A7</i>	0.36 0.04	1	0.82 <0.001	0.79 <0.001	0.40 0.02	0.60 <0.001	-0.36 0.04	*		0.46 0.01	0.42 0.01		0.41 0.02
<i>S100A8</i>		0.82 <0.001	1	0.98 <0.001	0.40 0.02	0.39 0.03	-0.61 <0.001	-0.46 0.01			0.60 <0.001	0.44 0.01	0.76 <0.001
<i>S100A9</i>	0.36 0.04	0.91 <0.001	0.95 <0.001	1	0.43 0.01	0.41 0.02	-0.62 <0.001	-0.48 0.004			0.58 <0.001	0.48 0.01	0.78 <0.001
<i>SPRR1A</i>		0.69 <0.001	0.76 <0.001	0.80 <0.001	1	*	*	*	0.48 0.005		0.36 0.04	0.35 0.05	*
<i>SPRR2C</i>	0.46 0.01	0.84 <0.001	0.62 <0.001	0.72 <0.001	0.56 <0.001	1				0.48 0.01			
<i>LOR</i>		-0.61 <0.001	-0.75 <0.001	-0.72 <0.001	-0.65 <0.001	-0.46 0.01	1	0.79 <0.001			-0.57 <0.001	-0.57 <0.001	-0.72 <0.001
<i>FLG</i>		-0.60 <0.001	-0.67 <0.001	-0.68 <0.001	-0.67 <0.001	-0.50 0.003	0.83 <0.001	1			-0.39 0.02	-0.48 0.005	-0.51 0.002
<i>IVL</i>			0.48 0.005						1			0.47 0.01	0.59 <0.001
<i>RPTN</i>										1			
<i>KRT6A</i>		0.48 0.004	0.56 <0.001	0.51 0.002	0.54 0.001		-0.37 0.03				1	0.62 <0.001	0.64 <0.001
<i>KRT6B</i>					0.44 0.01							1	0.60 <0.001
<i>KRT16</i>		0.56 <0.001	0.77 <0.001	0.76 <0.001	0.71 <0.001	0.44 0.01	-0.68 <0.001	-0.54 0.001	0.41 0.02		0.43 0.01	0.42 0.02	1

Pearson's correlation coefficients for each pair of genes in non-lesional (lower right part of the table, below the diagonal) and lesional skin (upper right part, above the diagonal) at p values <0.05 are shown. In lesional skin, a significant correlation, new in comparison to nonlesional skin (a correlation at p < 0.005–0.001, not observed in non-lesional skin) is marked by italics. Complete

loss of correlation (lack of significant correlation where it was significant in nonlesional skin) is marked by an asterisk. Please note that from 9 significant changes observed, 6 were noticed for *SPRR1A* transcript and 2 for *keratin 6B* transcript. *RPTN* = repetin; *KRT* = keratin.

and 0.79, respectively, p < 0.001). However, this correlation was much weaker at the low expression levels of both genes, seen more often in lesional skin (fig 2a). An inverse correlation with the *LOR* transcript level was noticed for *S100A8* and *A9* both in nonlesional and lesional skin; for *S100A7* and *SPRR1A*, such a correlation was noticed in nonlesional skin only.

Expression of Alternative Pathway Keratins

Keratins 6A, 6B and *16* showed a very distinct increase in mRNA in lesional skin (fold changes of 4.8, 2.1 and

13.5, respectively; table 2). Only *keratin 6A* expression was significantly correlated between nonlesional and lesional skin (R = 0.64, p < 0.001). In both nonlesional and lesional skin, *keratin 16* was correlated with *S100A7–A9* (the strongest correlation was noted for *S100A8*: R = 0.77, p < 0.001) and inversely correlated with *LOR* and *FLG* (fig. 2b, table 3); only for *SPRR1A* was the correlation lost in lesional skin. For *keratin 6B*, correlations were more significant in lesional skin, and 2 of them reached the level of p < 0.001.

Fig. 2. Correlations between expression levels of *LOR* and *FLG* (a) and *LOR* and *keratin 16* (b).

Fig. 3. Hierarchical clustering of genes in nonlesional (left panel) and lesional (right panel) skin. Note the changed position of *SPRR1A* and *SPRR2C*. KRT = Keratin; RPTN = repetin.

Hierarchical Clustering of Gene Expression Values in Lesional and Nonlesional Skin

Hierarchical clustering was carried out to analyze the pattern of similarity in gene expression in lesional and nonlesional skin and thus to identify gene clusters. In nonlesional AD skin, we found coexpression of *S100A7–A9*, *SPRR*, *IVL* and alternative pathway keratin genes (fig. 3). A moderate similarity was observed between

S100A2 and *repetin*. On the other hand, *LOR* and *FLG* transcripts formed a distinctly different cluster.

A quite different pattern was observed in lesional skin; *SPRR* genes lost the strong association with *S100* and *keratin* genes seen in nonlesional skin and appeared to be coregulated with *S100A2* (*SPRR1A*) and *repetin* (*SPRR2C*). Coexpression of *FLG* and *LOR* was retained in lesional skin.

Discussion

In the present study, we compared the expression pattern of EDC genes in lesional skin biopsies to that found in biopsies of nonlesional skin from the same AD patients, all taken contralaterally at the same location, at a sufficient distance from other lesions. Recently, it has been shown that a defective skin barrier, caused by loss-of-function mutations in the *FLG* gene, significantly predisposes individuals to AD. Unfortunately, the *FLG* genotype of our patients was unknown; thus, we were unable to assess the downstream effects of *FLG* loss-of-function mutations. Instead, we focused on other 1q21 genes which had previously been found to exhibit changes in expression by an AD microarray study [6], in order to validate the results by an independent approach and to investigate in a more detailed manner their expression pattern, so far uncharacterized. In chronic AD skin lesions, we observed a distinct rise in RNA encoding IVL and S100 calcium-binding proteins A2, A7, A8 and A9, as well as *SPRR* proteins 1A and 2C. The rise occurred in parallel to the overexpression of alternative pathway *keratins* 6A, 6B and 16. Among the closely correlated genes, a deregulation of *SPRR1A* was noticed; of 9 significant correlation changes (table 3), 6 affected this transcript. Also, a distinct change in the hierarchical clustering tree was observed for *SPRR1A* and, to a lesser extent, *SPRR2C* (fig. 3).

In intact human epidermis, the *SPRR1* and 2 proteins are cross-linked to LOR, together with keratins 1, 2 and 10 as well as *FLG* and elafin [2]. Upregulation of *SPRR1A* and 2C mRNA, found in AD lesions in our study, might partially compensate for the observed *LOR* transcript deficiency. This was seen in studies using *LOR*-deficient mice [2, 21], and was also accompanied by augmented IVL expression, similar to our human study. The induction of *SPRR1* and 2 gene expression in inflammatory skin diseases has been shown previously [22] and was interpreted as part of the response to conditions that require rapid epidermal regeneration. Expression of *SPRR* genes is believed to be of major importance for CE biomechanical properties [16, 23]. *SPRR2C* protein was previously described to be much more intensely upregulated in psoriasis than in AD [24] and is regarded as an epithelial host defense protein [6]. These changes need to be further verified at the protein level. Sugiura et al. [6] also found changes in other *SPRR* family transcripts, i.e. upregulation of *SPRR1B* and downregulation of *SPRR3*. Unfortunately, these transcripts were not included in our panel of studied genes.

Contrary to the conclusions of Sugiura et al. [6], we noticed a significant upregulation of *IVL* RNA in AD skin lesions. At the protein level, a decrease in IVL expression in AD skin lesions was reported in other studies [5, 14]. However, our results are consistent with those of Hirao et al. [13], who described a distinct but heterogeneous rise in IVL-containing immature CE in stratum corneum cells of lesional AD skin. IVL is regarded as a human keratinocyte differentiation marker [25], and its rise is an early event in normal epidermal differentiation. We conclude that this step of EDC differentiation is not impaired in AD. However, the *IVL* RNA increase was not accompanied by an increase in *LOR* RNA, as would be expected in normal skin; on the contrary, a significant decrease in *LOR* RNA was observed in our study. *LOR* is the major component of the cornified cell envelope. It is expressed in a keratinocyte- and differentiation-specific manner and is present only in terminally differentiated keratinocytes. Immunohistochemistry and Western blot analysis showed *LOR* expression to be upregulated 3-fold in both nonlesional and lesional AD skin when compared to normal controls [14]. In the same study, there was no difference in *LOR* transcript copy number at the mRNA level when lesional AD skin was compared to nonlesional AD skin. Our observation was different and remains in agreement with the cited microarray data [6]. It is also consistent with the downregulation of *LOR* described by Hohl [26], who also noticed the similarity between *FLG* and *LOR* expression changes. This change may be important in light of the well-known strict regulation of the *LOR* gene at the transcriptional level [15, 27]. In situ hybridization analyses have shown that *LOR* transcripts are localized only in the upper spinous and granular layers of the normal epidermis [28]. In vitro data [15] suggest a complex interplay between transcription factors of the Sp1, CREB and AP1/AP2 families in the regulation of the *LOR* gene. Until now, there have been no data regarding whether these mechanisms do operate in AD lesional skin. On the other hand, the observed decrease might be secondary to the high level of protein product [14].

Other CE genes, activated during normal development before *LOR* or simultaneously with its expression, were distinctly upregulated in lesional AD skin. The coordinated upregulation of *S100A7–A9* reported in our study confirms the data obtained by Broome et al. [29] in inflammatory disorders of the skin. The expression of *S100A7–A9* is low in normal epidermis. Their overexpression is typical for psoriasis; however, *S100A7* has been found in many other epidermal inflammatory diseases including AD and was proposed to function as a kerati-

nocyte-derived chemotactic agent [9]. Multiple immunological functions have been attributed to S100 proteins, as well as a role in epidermal wound repair, differentiation and the response to stress [30, 31].

We did not observe significant changes in *FLG* RNA in chronic AD skin lesions; however, our conclusion is weakened by the fact that our choice of primers for this transcript was suboptimal – both primers were located within exon 3 and led to the amplification of 2 amplicons of identical size. Nevertheless, the possible variability in the number of repeats in the *FLG* gene should not influence the comparison of *FLG* gene expression between affected and unaffected skin, as an equal number of repeats is expected in both specimens from one individual. As already mentioned, nothing is known in relation to the crucial question whether the changes in 1q21 gene expression observed in our study differ depending on the presence of germline *FLG* mutations predisposing to AD. However, it should be considered that the mutations in the *FLG* gene responsible for the development of AD in a significant number of patients may not be reflected by a decrease in *FLG* transcript itself. These mutations nearly always occur in the last exon of the gene, and the mutated transcript escapes the non-sense-mediated mRNA degradation pathway, while the decrease in protein expression is very distinct and related to the loss of translation or instability of the protein product [3, 4, 32].

Our study comprised only AD patients, without a healthy control group. Thus, the only comparison possible was the comparison between nonlesional and lesional AD skin. This model may potentially miss certain changes seen in AD patients even in nonlesional skin. It is well known that in AD patients, the *FLG* content of nonlesional skin is lower than in healthy persons; the same variation has been described for *LOR* and *IVL* [5, 14, 26]. These genes exhibited a strong correlation of expression

level between nonlesional and lesional skin, indicating indirectly that they were influenced by a genetic predisposition.

In light of the observed increase in the expression of alternative keratins, the changes seen in chronic AD skin lesions may be interpreted as the stress response of the epidermis in the context of high regeneration requirements; keratins 6 and 16 are markers of an alternative pathway of keratinocyte differentiation, a response to various conditions that require rapid epidermal regeneration and protective gene expression [33]. Sugiura et al. [6] were the first to document their upregulation in AD, concurrently with changes in the locus 1q21 genes.

An important finding of our study is the deregulated increase in *SPRR* expression in chronic atopic skin lesions; *SPRR1A* and (to a lesser extent) *SPRR2C* lose their coexpression with *S100* genes and other 1q21 transcripts. We hypothesize that this altered pattern reflects an insufficient rise in *SPRR* expression, unable to compensate for the lack of *LOR* and thus contributing to the persistence of chronic AD skin changes. According to Segre [16], it is necessary to elucidate the multiple ways in which keratinocytes signal a barrier disruption in AD lesions and maintain a stress response. Turning off the stress response in the skin may be regarded as a goal in the treatment of AD skin lesions, and *SPRR* genes might be the new targets of such an approach.

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