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ORIGINAL INVESTIGATION

Expression signature of epidermolysis bullosa simplex

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Abstract Epidermolysis bullosa simplex (EBS) is a skin disorder resulting from a weakened cytoskeleton of the proliferative compartment of the epidermis, leading to cell fragility and blistering. Although many mutations have been identified in intermediate filament keratins *KRT5* and *KRT14*, detailed pathogenic mechanisms and the way these mutations affect cell metabolism are unclear. Therefore, we performed genomic and transcriptomic study in six Canadian EBS patients and six healthy subjects. We first characterized these patients at the genetic level and identified six pathogenic mutations of which two were novel. Then, we performed an expression microarray analysis of the EBS epidermis tissue to identify potential regulatory pathways altered in this disease. Expression profiling comparisons show that 28 genes are differentially expressed in EBS patients compared to control subjects and 41 genes in severe phenotype patients (EBS-DM) compared to their paired controls. Nine genes involved in fatty acid metabolism and two genes in epidermal keratinization are common altered expressed genes (up regulated) between the two subgroups. These two biological pathways contribute both to the formation of the cell envelope barrier and seem to be defective in the severe EBS phenotype. This study identifies, for the first time, the fatty acid metabolism disruption in EBS.

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Introduction

Epidermolysis bullosa simplex (EBS) is a rare skin disease with a prevalence ranging from 1 in 30,000 to 1 in 50,000 individuals worldwide (Ciubotaru et al. 2003; Rugg et al. 2007). Several clinical variants of EB simplex have been described. The most frequent and widely known variants are: EB simplex-generalized (gen-nonDM; in which the distribution of blistering is "generalized" over the body), EB simplex Dowling-Meara (EBS-DM; in which blisters are also generalized but show a distinct "herpetiform" or clustered pattern), and EB simplex-localized (EBS-loc, in which the distribution of blistering is "localized", e.g., primarily restricted to hands and feet) (Fine et al. 2008). Histological and/or ultrastructural examinations of this disease show that blistering occurs in the subnuclear cytoplasm of basal keratinocytes (Fine et al. 2000).

Most cases of EBS are inherited in an autosomal dominant Mendelian model pattern and are caused by defects in the genes encoding the intermediate filament proteins keratin 5 (*KRT5*) and keratin 14 (*KRT14*) (Irvine and McLean 1999). More than 250 different pathogenic mutations have now been documented in these two genes (http://www.interfil.org). Although mutations in intermediate filament genes cause many human disorders, the pathomechanisms and the way these mutations affect cell metabolism remain unknown. More recently, gene expression studies in EBS mouse models showed an increase in proinflammatory cytokines and chemokines, which mediate an immune response, and an increased number of Langerhans cells (Lu et al. 2007; Roth et al.

2009). Also, many cell junction components were shown to be differentially expressed in cultured EBS cell lines (Liovic et al. 2009).

In this study, we performed a mutational analysis of six human EBS patients followed by a gene expression microarray analysis of their EBS epidermis tissue to examine the broad spectrum of effects of *KRT5* and *KRT14* mutations in vivo. The present study report is the first global expression study of human EBS epidermis tissue. It reveals that alteration of lipid metabolism and epidermis barrier function are major pathways disrupted in EBS, which may lead to new insights into epithelial biology in the context of genetic keratin diseases.

Materials and methods

Subjects

Six unrelated Canadian EBS patients were evaluated in this study. They were recruited from either of the two Canadian specialized clinics located in Montreal (Sainte-Justine Hospital) and Saguenay (Chicoutimi Hospital). Autosomal dominant mode of transmission of the disease was confirmed in two EBS patient families and all the other patients were the first case in their families. Patients were clinically diagnosed with EBS and classified into the corresponding subtypes. Two patients had the severe phenotype (EBS-DM) (EBS2 and EBS6) and all the others had a moderate phenotype (EBS-loc) (EBS1, EBS3, EBS4 and EBS5). These diagnoses were further confirmed by sequencing the *KRT5* and *KRT14* genes. Six healthy individuals were also recruited in this study and were paired to the EBS patients according to the age (±3 years) and gender to perform comparative microarray analysis. The inclusion criteria for the EBS patients included no genetic diseases other than EBS and no genetic dermatologic diseases for the controls. The ethic committees from both hospitals approved the study and all subjects (or their parents for affected children) gave their informed consent.

Sequence

Genomic DNA was extracted from peripheral blood lymphocytes using a DNA extraction kit QIAamp DNA Blood Midi kit (Qiagen, ON, Canada) according to the manufacturer's instructions. Total DNA of the patients—and their parents when available—was used as a template for amplification of the genomic sequences of *KRT5* and *KRT14*. *KRT5* segments (including nine exons and all exon–intron borders) and *KRT14* segments (including eight exons and all exon–intron borders) were amplified as previously described (Stephens et al. 1997; Schuilenga-Hut et al. 2003). Genomic DNA samples from 50 normal healthy individuals were used as controls. Sequence analyses were performed using Big Dye terminator technology (ABI 3730xl) (Applied Biosystems, ON, Canada) and were analyzed using Bioedit packages.

Sample tissue and RNA extraction

Superficial 2-mm punch biopsies of buttocks normal-appearing skin—composed of mainly epidermis with minimal amounts of dermis—were collected on the six EBS patients (two females and four males) and healthy volunteers. The biopsies were processed and the tissue samples were immediately placed in RNAlater (Applied Biosystems, ON, Canada) to stabilize intracellular RNA during transport. RNA was extracted in parallel from paired controls and EBS subjects. The samples (mean weight 11 mg) were mechanically homogenized (Rotor/Stator homogeniser, PowerGen) and total RNA was extracted using the RNEasy Fibrous Tissue Mini Kit (Qiagen, ON, Canada) according to the manufacturer's instructions. RNA integrity and yield were assessed using Agilent BioAnalyser RNA 2100 and by spectrophotometry.

Microarrays

Gene expression was assessed on the Affymetrix Genechip Human Gene 1.0 ST microarrays containing 28,869 oligonucleotide target probes. Hybridization and scanning of images were performed at McGill University and Genome Quebec Innovation Centre (http://www.genomequebec.mcgill.ca). To minimize technical variability, RNA processing steps (RNA extraction, probe labeling and chip hybridization) were performed in parallel for control and EBS samples. Detailed probe synthesis hybridization and washing protocol have been previously described (Novak et al. 2002). The scanned images were analyzed using the Microarray Analysis Suite 5.0 (MAS5, Affymetrix) and the raw image files obtained (CEL format) were used for statistical analysis. Analyses were performed with the Affy and Limma packages available in Bioconductor (http://www.bioconductor.org), which use R language (http://www.R-project.org) (Gautier et al. 2004; Smyth 2004). Quality tests to look at artifact or variability among microarrays were done with the Affy package. Probe intensities from the 12 chips were first normalized using robust multi-array analysis (RMA), which comprises background correction, quantile normalization and median polish steps. Use of the RMA method in conjunction with p value and fold change cutoff (p value <0.05 and absolute fold change >2) allowed us to avoid performing multi-testing correction (Gregory Alvord et al. 2007; Bolstad et al. 2003). To even better decrease false discovery rate,

Smyth's moderated *t* test was used instead of the standard *t* test (Limma package) (Gregory Alvord et al. 2007).

We performed three types of gene expression profiling comparisons by taking into account the severity of the phenotype; we compared the gene expression patterns between the six EBS patients and the six paired controls, between the two EBS-DM patients and their respective controls, and finally between the four EBS-loc patients and their respective controls. The data of the expression arrays produced for this report have been deposited in the Gene Expression Omnibus databank (http://www.ncbi.nlm.nih.gov/geo).

qRT-PCRs

To validate differences in gene expression levels, qRT-PCR was performed on a selected set of genes. Seven genes [acyl-CoA wax alcohol acyltransferase 2 (*AWAT2*), diacylglycerol *O*-acyltransferase 2-like 6 (*DGAT2L6*), elongation of very long-chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 3 (*ELOVL3*), fatty acid desaturase 2 (*FADS2*), acyl-CoA synthetase bubblegum family member 1 (*ACSBG1*), small proline-rich protein 4 (*SPRR4*) and keratin 79 (*KRT79*)] were selected to be tested using real-time PCR based on their biological function. First, RNA integrity was evaluated using capillary electrophoresis. Superscript III (Invitrogen, Burlington, ON, Canada) with oligodT(20) primers was used for cDNA synthesis. The RT-PCR reaction was performed using TaqMan technology in the Rotor-Gene 6000 (Corbett Research, Sydney, Australia) with 0.45 ng of cDNA, TaqMan primers and master mix (Applied Biosystems, ON, Canada) for a final volume of 10 µl. Each sample was run twice in triplicate with a negative control and the results were averaged for final RNA quantification. A standard curve was done with three serial dilutions in triplicate for each selected gene and for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), which was used as a housekeeping gene. Quantification obtained from standard curves of each gene was normalized to the relative amount of *GAPDH* using the two standard curves method implemented in the Rotor-Gene 6 software (version 6.0). Measures of expression for the two phenotypes expressed as mean ± SEM were compared by Student's bilateral *t* test. A p value <0.05 was considered to be significant.

Western blotting

ELOVL3, one of the altered expression genes between EBS patients and controls, was selected to document its protein level and to compare it with the difference in gene expression observed. As it remained skin tissues only from one EBS patient and one healthy control, we extracted proteins from these two biopsies. The samples were mechanically homogenized (Rotor/Stator homogeniser, PowerGen) and total proteins were extracted using the RNEasy Mini Kit (Qiagen, ON, Canada) according to the manufacturer's instructions. Quantifications were carried out using the Bradford method. Proteins were separated on SDS-polyacrylamide gel with 50 µg of the samples. Gel was transferred to nitrocellulose membrane and transfer efficiency was controlled by Ponceau Red staining. The membrane was blocked with TBS Blotto A Blocking Reagent. ELOVL3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used at a dilution of 1:100 and β-actin (Chemicon, ON, Canada) as loading control, at a dilution of 1:500. Bound primary antibodies were revealed using peroxidase-conjugated secondary antibodies and specific immuno-complexes were detected using an ECL Western blotting detection kit. Quantification of signal intensities was performed using Image J software.

Results

Two novel mutations identified in Canadian EBS patients

The studied patients had been clinically classified into two EBS subtypes; EBS-loc (EBS1, EBS3, EBS4 and EBS5) and EBS-DM (EBS2, and EBS6). We identified six pathogenic mutations from which two are novel (Fig. 1). Three mutations reside in *KRT5* and three reside in the *KRT14* gene, including five missense mutations and one nonsense mutation. A number of additional single nucleotide polymorphisms (SNPs) in *KRT5* and *KRT14* were also identified in the patients, as outlined in Table 1.

Four recurrent mutations

We identified the nucleotide change 1130 T>C within *KRT14* in EBS1 patient (EBS-loc), which introduces the amino acid replacement I377T (isoleucine to threonine) lying close to the stutter region in helix 2B of the protein.

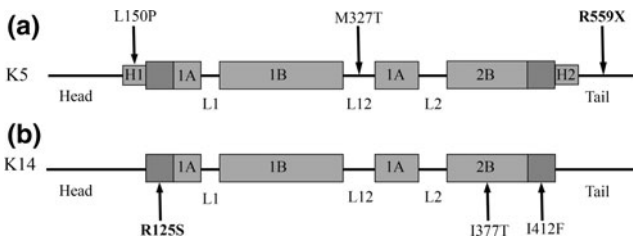

Fig. 1 Schematic representation of identified mutation locations in keratin 5 (K5) and in keratin 14 (K14) proteins. **a** Mutations within K5 are L150P, M327T, and R559X. **b** Mutations within K14 are R125S, I377T, and I412F. Novel mutations are indicated in bold

Table 1 Epidermolysis bullosa simplex subtypes and mutations

Patient	Gender	Age (years)	EBS subtype	Gene	Mutation	Effect	Domain	References	*KRT5* SNPs	*KRT14* SNPs
EBS1	♂	31	EBS-loc	*KRT14*	1130T>C	I377T	2B	Rugg et al. 2007	rs17852231, rs1132948, rs4761924, rs11549950, rs11549949	rs6503639, rs3826550
EBS2	♂	8	EBS-DM	*KRT5*	449T>C	L150P	H1	Oh et al. 2007	rs11549951, rs17852231, rs1132948, rs11549950, rs11549949	rs11551759, rs3826551, rs6503639, rs3826550, rs38265489
EBS3	♂	36	EBS-loc	*KRT5*	980T>C	M327T	L1-2	Humphries et al. 1996; Chan et al. 1994	rs11549951, rs641621, rs641615, rs11266758, rs113294, rs4761924, rs11549950, rs11549949	rs11551759, rs11551758, rs3826551, rs6503639, rs3826550, rs3826549, rs9915113
EBS4	♀	7	EBS-loc	*KRT14*	1234A>T	I412F	2B	Bolling et al. 2011	rs638907, rs641621, rs641615	rs11551759, rs11551758, rs3826551, rs6503639, rs3826550, rs3826549, rs9915113, rs11551760
EBS5	♀	51	EBS-loc	*KRT5*	1675C>T	R559X	Tail	Novel	No SNPs	Not performed
EBS6	♂	25	EBS-DM	*KRT14*	373C>A	R125S	1A	Novel	No SNPs	rs11551758, rs3826551, rs6503639, rs3826550, rs3826549

♂ Male
♀ Female

This mutation was previously reported by Rugg and colleagues in one Scotland EBS-loc patient (Rugg et al. 2007). In EBS2 patient (EBS-DM), we identified the nucleotide variation 449T>C in *KRT5*, which results in the amino acid substitution L150P (leucine to proline) in the H1 domain of the protein. This variation had been previously described in one non-Caucasian gen-nonDM patient (Oh et al. 2007). We identified a 980T>C nucleotide variation in *KRT5* in an EBS3 patient (EBS-loc), which resulted in the amino acid substitution p.M327T (methionine to threonine) in the L12 linker region of the protein. This variation was already reported twice in association with EBS-loc (Chan et al. 1994; Humphries et al. 1996). The I412F missense mutation (isoleucine to phenylalanine) was identified in the EBS4 patient (EBS-loc). It lay in the 2B domain of K14 protein and caused by the nucleotide substitution 1234A>T. This mutation was recently described in an EBS-loc patient (Bolling et al. 2011).

Two novel mutations

A novel missense mutation was found in the *KRT14* gene at nucleotide position 373C>A in the EBS6 patient (EBS-DM) resulting in the novel variation (R125S) (arginine to serine) in the 1A domain of the protein. No such mutation was found in 50 unrelated controls. In *KRT5*, we identified the novel nucleotide sequence variation 1675C>T in EBS5 patient (EBS-loc) that resulted in a truncated protein at the tail domain (R559X).

Skin lipid metabolism and keratinization pathways

We report the results of expression microarray studies using tissues obtained from skin biopsies of six EBS patients and from six paired healthy controls (Fig. 2). We identified 28 genes that showed significant differences in expression (absolute fold change >2 and p value <0.05) in EBS subjects compared to unaffected ones. These genes were classified into five different biological pathways [acid fatty metabolism (nine genes), keratinization (three genes), cell growth and proliferation (eight genes), immune response (two genes), and others functions (six genes)] (Table 2). When comparing only the expression profiling of EBS-DM patients (EBS2 and EBS6) to their paired controls, we found 41 altered expressed genes that were all expressed higher in EBS-DM patients. These genes could be clustered in the same biological processes as the previous expression pattern comparison (acid fatty metabolism (eighteen genes), keratinization (ten genes), cell growth and proliferation (five genes), immune response (three genes) and others functions (five genes) (Table 3). Nine genes involved in fatty acid metabolism (*AWAT2*, *DGAT2L6*, *ELOVL3*, *THRSP*, *FADS2*, *FAR2*, *ACSBG1*, *AADACL3* and *CRAT*) and two genes in

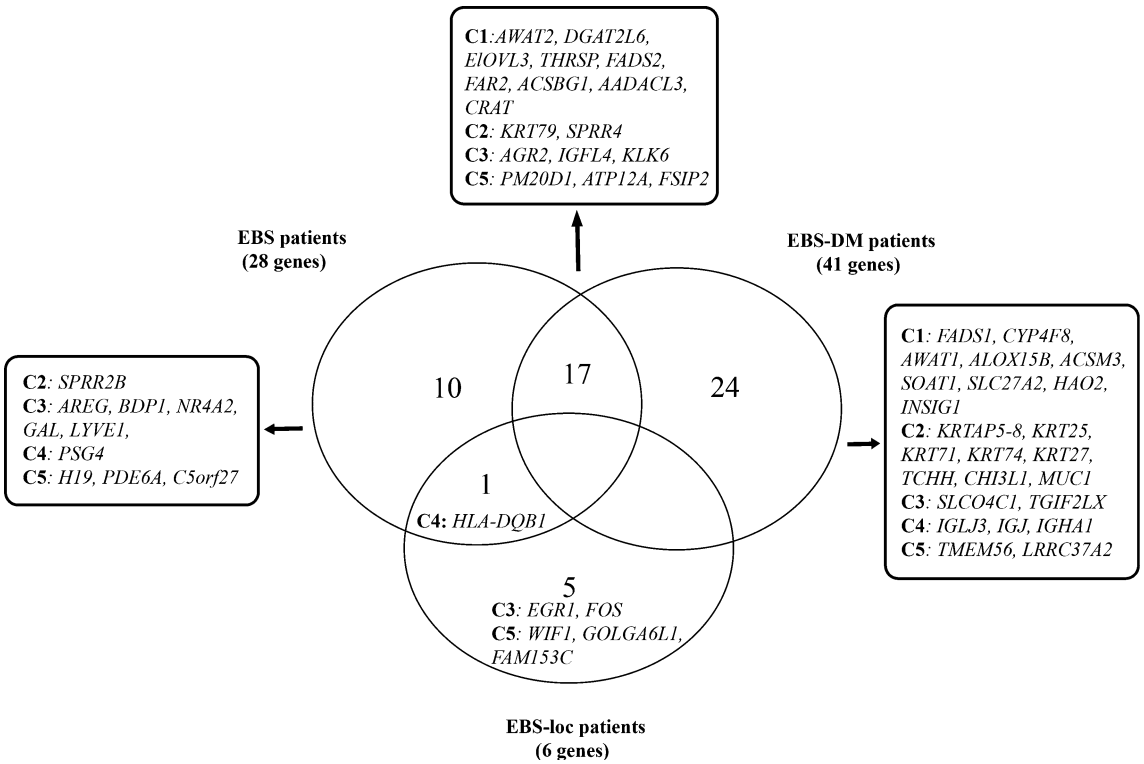

Fig. 2 Common and unique altered expressed genes between the three epidermolysis bullosa simplex subgroups: microarrays data showed that 28 were significantly altered expressed genes between the six EBS and the six control subjects, 41 genes between the two EBS-DM and their two paired controls, and six genes between the four EBS-loc patients and their control subjects. Seventeen genes are common altered expressed genes between EBS and EBS-DM patients among them nine genes are involved in fatty acid metabolism (*AWAT2*, *DGAT2L6*, *ELOVL3*, *THRSP*, *FADS2*, *FAR2*, *ACSBG1*, *AADACL3*, and *CRAT*) and 2 genes (*SPRR4* and *KRT79*) in epidermal keratinisation). Only one common gene between EBS and EBS-loc patients (*HLA-DQB1*). *C1* Lipid biosynthetic process genes cluster. *C2* Keratinisation genes cluster. *C3* Cell growth and proliferation genes cluster. *C4* Immune response genes cluster. *C5* Other functions genes cluster

epidermal keratinization (*SPRR4* and *KRT79*) are common altered expressed genes between the EBS and EBS-DM subgroups. On comparing the profiling expression of the EBS-loc patients (EBS2, EBS3, EBS4, and EBS5) to their paired controls, we found that the expression of only six genes is modulated in these patients [cell growth and proliferation (two genes), immune response (one gene), and others functions (three genes)] (Table 4).

To validate the microarray data, we selected seven of the differentially expressed genes (*AWAT2*, *DGAT2L6*, *ELOVL3*, *FADS2*, *ACSBG1*, *SPRR4*, and *KRT79*) from the two lipid metabolism and skin keratinization categories to be tested using real-time PCR, which usually confirms about 87% of microarray results done using RMA analyses (Dallas et al. 2005). These genes were selected according to known functions suggesting a possible involvement in EBS pathogenesis, although no possible difference of expression linked to EBS disease has yet been documented. Real-time PCR showed higher expression of all seven genes in EBS subjects and confirmed the significant difference in gene expression ($p < 0.05$) for five genes (*AWAT2*, *DGAT2L6*, *ELOVL3*, *FADS2*, and *SPRR4*) between EBS-DM patients and their paired controls (Fig. 3). Limited by the RNA quantity available, we could not validate all differences observed in the microarray results.

Protein expression

Protein level for one of the key identified fatty acid metabolism genes (*ELOVL3*) was also then evaluated by immunoblotting. Figure 4 shows the results of immunoblot analysis, demonstrating a higher expression of this protein in EBS patient than the wild-type control.

Discussion

Different degrees of clinical severity of EBS arise from mutations occurring in different parts of the keratin molecule (Chamcheu et al. 2009). The most disruptive mutations are those within the helix boundary motifs at either end of the rod domain of the proteins (the helix initiation peptide of the 1A segment and the helix termination peptide of the 2B segment), whereas mutations associated with

Table 2 List of genes differentially expressed in EBS skin subjects in comparison with healthy controls

Clusters	Probe set	ACCNUM	Gene symbol[a]	Gene name	Cytoband[b]	*p*	Flc[c]	Function[d]
Cluster 1: lipid biosynthetic process								
	8173349	NM_001002254	**AWAT2*	Acyl-CoA wax alcohol acyltransferase 2	Xq13.1	0.015	7.58	Esterification of long-chain (wax) alcohols with acyl-CoA-derived fatty acids to produce wax esters (Turkish et al. 2005)
	8168098	NM_198512	**DGAT2L6*	Diacylglycerol *O*-acyltransferase 2-like 6	Xq13.1	0.032	6.25	Triacylglyceride (DGAT) biosynthesis (Holmes 2010)
	7930025	NM_152310	**ELOVL3*	Elongation of very long-chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 3	10q24.32	0.029	4.1	Elongation of saturated fatty acyl-CoAs into very long-chain fatty acids (Westerberg et al. 2006)
	7942793	NM_003251	*THRSP*	Thyroid hormone responsive	11q13.5	0.029	3.95	Lipogenesis (Moreau et al. 2009)
	7940565	NM_004265	**FADS2*	Fatty acid desaturase 2	11q12.2	0.011	3.81	Catalyzation of biosynthesis of highly unsaturated fatty acids (Ge et al. 2003)
	7954631	NM_018099	*FAR2*	Fatty acyl-CoA reductase 2	12p11.22	0.035	2.61	Catalyzation of the reduction of fatty acyl-CoA to fatty alcohols (Cheng and Russell 2004)
	7990683	NM_015162	**ACSBG1*	Acyl-CoA synthetase bubblegum family member 1	15q23-q24	0.042	2.53	Activation of long-chain fatty acids for both synthesis of cellular lipids, and degradation via beta-oxidation (Steinberg et al. 2000)
	7897960	NM_001103170	*AADACL3*	Arylacetamide deacetylase-like 3	1p36.21	0.042	3.8	Hydrolase activity, metabolic process (Gregory et al. 2006)
	8164535	NM_000755	*CRAT*	Carnitine *O*-acetyltransferase	9q34.1	0.034	2.33	Transport of acetyl-CoA into mitochondria (Wu et al. 2003)
Cluster 2: keratinization								
	7963545	NM_175834	**KRT79*	Keratin 79	12q13.13	0.027	3.53	Structural molecule activity, keratin filament (Rogers et al. 2005)
	7905536	NM_173080	**SPRR4*	Small proline-rich protein 4	1q21.3	0.015	2.87	Cross-linked envelope protein of keratinocytes (Cabral et al. 2001)
	7920201	NM_001017418	*SPRR2B*	Small proline-rich protein 2B	1q21-q22	0.026	2.71	Cross-linked envelope protein of keratinocytes (Cabral et al. 2001)
Cluster 3: cell growth and proliferation								
	8138381	NM_006408	*AGR2*	Anterior gradient homolog 2 (*Xenopus laevis*)	7p21.3	0.003	2.02	May play a role in cell migration, cell differentiation and cell growth (Wang et al. 2008)
	8095744	NM_001657	*AREG*	Amphiregulin	4q13-q21	0.011	2.6	Promotion of the growth of normal epithelial cells and inhibition of the growth of certain aggressive carcinoma cell lines (Plowman et al. 1990)
	8106025	NM_018429	*BDP1*	B double prime 1, subunit of RNA polymerase III transcription initiation factor IIIB	5q13	0.009	2.12	Transcription from all three types of polymerase III promoters (Schramm et al. 2000)
	8055952	NM_006186	*NR4A2*	Nuclear receptor subfamily 4, group A, member 2	2q22-q23	0.024	2.27	Steroid hormone receptor activity, regulation of transcription (Martinat et al. 2006)

Table 2 continued

Clusters	Probe set	ACCNUM	Gene symbol[a]	Gene name	Cytoband[b]	*p*	Flc[c]	Function[d]
	7942064	NM_015973	*GAL*	Galanin prepropeptide	11q13.3	0.048	3.43	Proliferation. inflammatory response (Bauer et al. 2008)
	7946579	NM_006691	*LYVE1*	Lymphatic vessel endothelial hyaluronan receptor 1	11p15	0.004	–2.04	Autocrine regulation of cell growth (Banerji at al. 1999)
	8037750	NM_001002923	*IGFL4*	IGF-like family member 4	19q13.32	0.023	2.14	Cellular energy metabolism, growth and development (Emtage et al. 2006)
	8038683	NM_002774	*KLK6*	Kallikrein-related peptidase 6	19q13.3	0.034	2.5	Regulation of cell differentiation; serine-type endopeptidase activity (Iwata et al. 2003)
Cluster 4: immune response								
	8125461	AK097297	*HLA-DQB1*	Major histocompatibility complex. class II. DQ beta 1	6p21.3	0.018	2.69	MHC class II receptor activity (Jonsson et al. 1987)
	8037283	NM_002780	*PSG4*	Pregnancy-specific beta-1-glycoprotein 4	19q13.2	0.026	−2.12	Defense response (Teglund et al. 1994)
Cluster 5: other functions								
	7923837	NM_152491	*PM20D1*	Peptidase M20 domain containing 1	1q32.1	0.03	7.41	Metallopeptidase activity (http://www.uniprot.org)
	7945680	NR_002196	*H19*	H19. imprinted maternally expressed transcript (non-protein coding)	11p15.5	0.001	−2.59	Non-protein coding (http://www.uniprot.org)
	8115041	NM_000440	*PDE6A*	Phosphodiesterase 6A. cGMP-specific. rod. alpha	5q31.2-q34	0.046	2.35	3′,5′-cyclic-GMP phosphodiesterase activity. visual perception (Huang et al. 1995)
	7968062	NM_001676	*ATP12A*	ATPase. H^+/K^+ transporting. nongastric. alpha polypeptide	13q12.1-q12.3. 13q12.12	0.019	2.36	Catalyzation of the hydrolysis of ATP, responsible for potassium absorption in various tissues (Modyanov et al. 1991)
	8046824	AK092099	*FSIP2*	Fibrous sheath interacting protein 2	2q32.1	0.007	2.04	Protein binding (http://www.uniprot.org)
	8106999	NR_026936	*C5orf27*	Chromosome 5 open reading frame 27	5q15	0.014	2.08	Putative uncharacterized protein C5orf27 (http://www.uniprot.org)

[a] Genes marked by an asterisk were selected to be tested by real-time RT-PCR

[b] Gene location obtained from National Center for Biotechnology Information public database (http://www.ncbi.nlm.nih.gov)

[c] Fold changes (Flc) are indicated for each probe set significantly under- or over-expressed ($p < 0.05$; absolute Flc > 2) by epidermal cells of EBS patients compared with control subjects. Positive data indicate genes that are overexpressed; negative data indicate genes that are underexpressed by epidermal cells of EBS patients

[d] References that allow classification of differentially expressed genes in function categories

EBS gen-nonDM are located more centrally in the rod domain. Mutations in EBS-loc are most frequently found in the linker region L12, in the H1 domain of K5 or in the 2B segment of K14 (Szeverenyi et al. 2008). The distribution of our identified mutations on K5 and K14 is in favor of this observation (Fig. 1). However, the L150P missense mutation identified in the EBS2 patient showed an unusual genotype–phenotype correlation. The proband shows generalized blisters despite the mutation being in the non-helical head region, which is usually associated with EBS-loc. This is the first missense mutation report of an EBS-DM phenotype in the H1 domain of the K5 protein and outside the alpha-helical rod domain. This finding promotes the hypothesis that other factors influence the disease severity. In patient with EBS6 (EBS-DM), we identified the novel mutation R125S in K14. The arginine at position 125 in K14 (R125) is responsible for most cases of severe EBS-DM (Coulombe et al. 1991). R125 has been

Table 3 List of genes differentially expressed in EBS-DM skin subjects in comparison with healthy controls

Clusters	Probe set	ACCNUM	Gene symbol	Gene name	Cytoband	*p*	Flc	Function[a]
Cluster 1: lipid biosynthetic process								
	8173349	NM_001002254	*AWAT2*	Acyl-CoA wax alcohol acyltransferase 2	Xq13.1	0	10.41	Esterification of long-chain (wax) alcohols with acyl-CoA-derived fatty acids to produce wax esters (Turkish et al. 2005)
	8168098	NM_198512	*DGAT2L6*	Diacylglycerol *O*-acyltransferase 2-like 6	Xq13.1	0	7.12	Triacylglyceride (DGAT) biosynthesis (Holmes 2010)
	7930025	NM_152310	*ELOVL3*	Elongation of very long-chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 3	10q24.32	0	5.51	Eongation of saturated fatty acyl-CoAs into very long-chain fatty acids (Westerberg et al. 2006)
	7942793	NM_003251	*THRSP*	Thyroid hormone responsive	11q13.5	0	4.99	Lipogenesis (Moreau et al. 2009)
	7940565	NM_004265	*FADS2*	Fatty acid desaturase 2	11q12.2	0	4.09	Catalyzation of biosynthesis of highly unsaturated fatty acids (Ge et al. 2003)
	7948612	NM_013402	*FADS1*	Fatty acid desaturase 1	11q12.2-q13.1	0	3.73	Catalyzation of biosynthesis of highly unsaturated fatty acids (Leonard et al. 2000)
	8026442	NM_007253	*CYP4F8*	Cytochrome P450, family 4, subfamily F, polypeptide 8	19p13.1	0	3.52	Hydroxylation of arachidonic acid (20:4n-6) to (18R)-hydroxyarachidonate (Stark et al. 2006)
	8168107	NM_001013579	*AWAT1*	Acyl-CoA wax alcohol acyltransferase 1	Xq13.1	0	3.28	Esterification of long-chain (wax) alcohols with acyl-CoA-derived fatty acids to produce wax esters (Turkish et al. 2005)
	8004784	NM_001141	*ALOX15B*	Arachidonate 15-lipoxygenase, type B	17p13.1	0	3.16	Conversion of arachidonic acid exclusively to 15S-hydroperoxyeicosatetraenoic acid (Brash et al. 1997)
	8164535	NM_000755	*CRAT*	Carnitine *O*-acetyltransferase	9q34.1	0.001	2.7	Transport of acetyl-CoA into mitochondria (Wu et al. 2003)
	7993756	NM_005622	*ACSM3*	Acyl-CoA synthetase medium-chain family member 3	16p13.11	0	2.44	CoA ligase activity with broad substrate specificity (http://www.uniprot.org)
	7954631	NM_018099	*FAR2*	Fatty acyl-CoA reductase 2	12p11.22	0.002	2.43	Catalyzation of the reduction of fatty acyl-CoA to fatty alcohols (Cheng and Russell 2004)
	7907702	NM_003101	*SOAT1*	Sterol *O*-acyltransferase 1	1q25	0	2.38	Catalyzation of the formation of fatty acid-cholesterol esters, a role in lipoprotein assembly and dietary cholesterol absorption (Puglielli et al. 2001)
	7990683	NM_015162	*ACSBG1*	Acyl-CoA synthetase bubblegum family member 1	15q23-q24	0.002	2.37	Activation of long-chain fatty acids for both synthesis of cellular lipids, and degradation via beta-oxidation (Steinberg et al. 2000)

Table 3 continued

Clusters	Probe set	ACCNUM	Gene symbol	Gene name	Cytoband	*p*	Flc	Function[a]
	7983650	NM_003645	*SLC27A2*	Solute carrier family 27 (fatty acid transporter), member 2	15q21.2	0.001	2.21	Translocation of long-chain fatty acids (LFCA) across the plasma membrane (Schaffer et al. 1994)
	7904396	NM_001005783	*HAO2*	Hydroxyacid oxidase 2 (long chain)	1p13.3-p13.1	0	2.05	Catalyzation of the oxidation of L-alpha-hydroxy acids (Jones et al. 2000)
	8137526	NM_005542	*INSIG1*	Insulin-induced gene 1	7q36	0	2.04	Feedback control of cholesterol synthesis (Yang et al. 2002)
	7897960	NM_001103170	*AADACL3*	Arylacetamide deacetylase-like 3	1p36.21	0	4.4	Hydrolase activity, metabolic process (Gregory et al. 2006)
Cluster 2: keratinization								
	7905536	NM_173080	*SPRR4*	Small proline-rich protein 4	1q21.3	0	5.2	Cross-linked envelope protein of keratinocytes (Cabral et al. 2001)
	8180254	NM_021046	*KRTAP5-8*	Keratin-associated protein 5-8	11q13.4	0.001	5.04	Formation of a rigid and resistant hair shaft (Yahagi et al. 2004)
	7963545	NM_175834	*KRT79*	Keratin 79	12q13.13	0	4.92	Structural molecule activity, keratin filament (Wu et al. 2003)
	8015070	NM_181534	*KRT25*	Keratin 25	17q21.2	0	3.98	Essential for the proper assembly of type I and type II keratin protein complexes and formation of keratin intermediate filaments (Rogers et al. 2004)
	7963438	NM_033448	*KRT71*	Keratin 71	12q13.13	0	3.71	Essential component of keratin intermediate filaments in the inner root sheath (Ge et al. 2003)
	7963448	NM_175053	*KRT74*	Keratin 74	12q13.13	0	2.26	Specific component of keratin intermediate filaments in the inner root sheath (Ge et al. 2003)
	8015087	NM_181537	*KRT27*	Keratin 27	17q21.2	0	2.22	Essential for the proper assembly of type I and type II keratin protein complexes and formation of keratin intermediate filaments in the inner root sheath (Rogers et al. 2004)
	7920141	NM_007113	*TCHH*	Trichohyalin	1q21.3	0	2.08	Organization of the cell envelope (Steinert et al. 2003)
	7923547	NM_001276	*CHI3L1*	Chitinase 3-like 1 (cartilage glycoprotein-39)	1q32.1	0	2.22	Carbohydration-binding lectin with a preference for chitin (Renkema et al. 1998)
	7920642	NM_001018016	*MUC1*	Mucin 1, cell surface associated	1q21	0.003	2.46	Protective layer on epithelial cells against bacterial and enzyme attack (Yamamoto et al. 1997)
Cluster 3: cell growth and proliferation								
	8138381	NM_006408	*AGR2*	Anterior gradient homolog 2 (*Xenopus laevis*)	7p21.3	0.001	2.24	May play a role in cell migration, cell differentiation and cell growth (Wang et al. 2008)

Table 3 continued

Clusters	Probe set	ACCNUM	Gene symbol	Gene name	Cytoband	*p*	Flc	Function[a]
	8038683	NM_002774	*KLK6*	Kallikrein-related peptidase 6	19q13.3	0.001	4.36	Regulation of cell differentiation. serine-type endopeptidase activity (Iwata et al. 2003)
	8113369	NM_180991	*SLCO4C1*	Solute carrier organic anion transporter family, member 4C1	5q21.2	0	3.6	Sperm maturation by enabling directed movement of organic anions and compounds within or between cells (Chu et al. 2007)
	8168646	NM_138960	*TGIF2LX*	TGFB-induced factor homeobox 2-like, X-linked	Xq21.31	0.002	2.25	May have a transcription role in testis (http://www.uniprot.org)
	8037750	NM_001002923	*IGFL4*	IGF-like family member 4	19q13.32	0.002	3.63	Cellular energy metabolism, growth and development (Emtage et al. 2006)
Cluster 4: immune response								
	7981730	AB001736	*IGLJ3*	Immunoglobulin lambda joining 3	22q11.2	0.002	2.4	Immune response (García Miranda et al. 1983)
	8100827	NM_144646	*IGJ*	Immunoglobulin J polypeptide, linker protein for immunoglobulin alpha and mu polypeptides	4q21	0.003	5.97	Link of two monomer units of either IgM or IgA (http://www.uniprot.org)
	7981722	AK128476	*IGHA1*	Immunoglobulin heavy constant alpha 1	14q32.33	0	3.17	Immune response (Hobart et al. 1981)
Cluster 5: other functions								
	7903162	NM_152487	*TMEM56*	Transmembrane protein 56	1p21.3	0.001	2.2	Hypothetical protein (http://www.uniprot.org)
	7923837	NM_152491	*PM20D1*	Peptidase M20 domain containing 1	1q32.1	0	9.1	Metallopeptidase activity (http://www.uniprot.org)
	7968062	NM_001676	*ATP12A*	ATPase, H+/K + transporting, nongastric, alpha polypeptide	13q12.1-q12.3, 13q12.12	0.003	3.37	Catalyzation of the hydrolysis of ATP, responsible for potassium absorption in various tissues (Modyanov et al. 1991)
	8046824	AK092099	*FSIP2*	Fibrous sheath interacting protein 2	2q32.1	0.001	2.49	Protein binding (http://www.uniprot.org)
	8016259	NM_001006607	*LRRC37A2*	Leucine rich repeat containing 37, member A2	17q21.31	0	2.08	Protein binding (http://www.uniprot.org)

[a] References that allow classification of differentially expressed genes in function categories

shown to be replaced most often by histidine (R125H), cysteine (R125C) and glycine (R125G) (Stephens et al. 1997). The novel mutation R125S in patient EBS6 had never been described. In silico, using the NetPhos-2.0 server (http://www.cbs.dtu.dk), we found that the confidence for this being a phosphorylation site is quite low (data not shown).

Our mutational analysis showed a quiet genotype–phenotype relationship and a high rate of de novo mutations (EBS2, EBS4, EBS5, and EBS6), as the corresponding mutations were absent in the parents' peripheral blood cell DNA and/or there was no family history of blistering diseases. Thus, the position of the mutations can predict the clinical severity of EBS disease, and mutation analysis of *KRT5* and *KRT14* in EBS could be useful to insure precise diagnosis, prognostication, genetic counseling, prenatal diagnosis and identification of future gene therapy trials that might benefit patients.

In EBS patients, although many mutations have been identified in intermediate filament keratins *KRT5* and *KRT14* genes, detailed pathogenic mechanisms and the way these mutations affect cell metabolism are unclear. The current study shows for the first time the distinctive

Table 4 List of genes differentially expressed in EBS-loc skin subjects in comparison with healthy controls

Clusters	Probe set	ACCNUM	Gene symbol	Gene name	Cytoband	*p*	Flc	Function[a]
Cluster 1: lipid biosynthetic process								
Cluster 2: keratinization								
Cluster 3: cell growth and proliferation								
	8108370	NM_001964	Early growth response 1	*EGR1*	5q31.1	0.001	2.18	Regulation of transcription (Virolle et al. 2003)
	7975779	NM_005252	FBJ murine osteosarcoma viral oncogene homolog	*FOS*	14q24.3	0	2	Role in signal transduction, cell proliferation and differentiation (Okazaki and Sagata 1995)
Cluster 4: immune response								
	8125461	AK097297	Major histocompatibility complex, class II, DQ beta 1	*HLA-DQB1*	6p21.3	0	4.46	MHC class II receptor activity (Jonsson et al. 1987)
Cluster 5: other functions								
	7964722	NM_007191	WNT inhibitory factor 1	*WIF1*	12q14.3	0.001	−2.58	Signal transduction (Hsieh et al. 1999)
	7986598	NM_001001413	Golgin A6 family-like 1	*GOLGA6L1*	15q11.2	0.002	−2.42	Hypothetical protein (http://www.uniprot.org)
	8110417	NM_001079527	Family with sequence similarity 153, member C	*FAM153C*	5q35.3	0.004	2.48	Belongs to the FAM153 family (by similarity) (http://www.uniprot.org)

[a] References that allow classification of differentially expressed genes in function categories

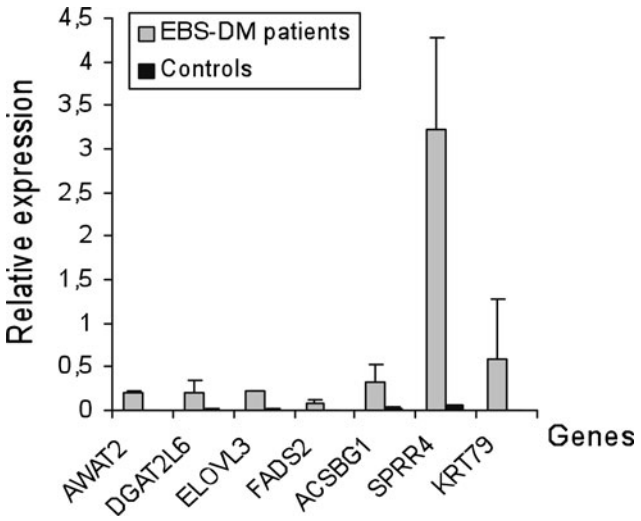

Fig. 3 Expression of the seven selected genes using real-time RT-PCR. Acyl-CoA wax alcohol acyltransferase 2 (AWAT2), diacylglycerol *O*-acyltransferase 2-like 6 (DGAT2L6), elongation of very longchain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 3 (ELOVL3), fatty acid desaturase 2 (FADS2), acyl-CoA synthetase bubblegum family member 1 (ACSBG1), small proline-rich protein 4 (SPRR4), and keratin 79 (KRT79) mRNA was extracted from skin biopsies of EBS-DM (*gray bars*) and paired controls (*black bars*) subjects. Measure of the mRNA expression by real-time RT-PCR was done twice in triplicate with negative control and normalized to GAPDH expression using two-standard curves method ($n = 4$). Data are expressed as mean + SEM values. AWAT2, DGAT2L6, ELOVL3, FADS2, and SPRR4 mRNA are significantly ($p < 0.05$) higher expressed in epidermis skin of EBS-DM subjects compared with controls

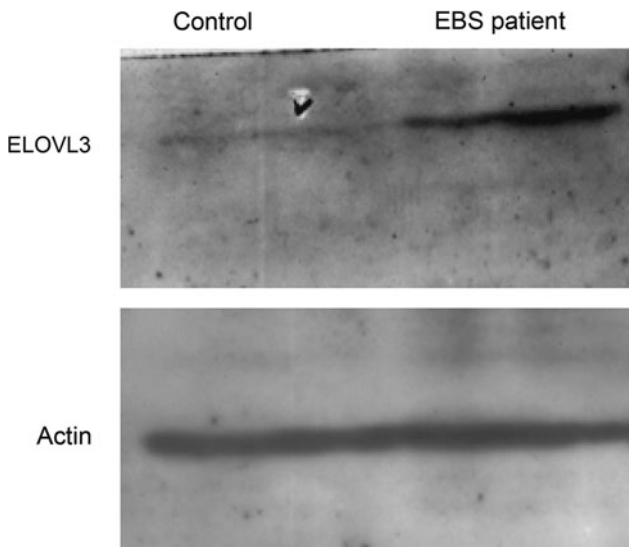

Fig. 4 The protein level of elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 3 (ELOVL3) in one EBS patient and one control. Protein expression was monitored in skin tissue lysates (50 μg total protein), separated on SDS-acrylamide gel and blotted onto nitrocellulose membrane with antibody against ELOVL3. *β*-Actin was used as loading control

pattern of gene expression that characterizes EBS versus healthy skin tissue and provides a potential signature for identification of the biological pathways altered in this disease.

Our microarrays data from the six human EBS skin biopsies and the control subjects showed that two major biological processes present higher expression levels in the skin of the EBS patients, which are the lipid metabolism (nine genes) and the epidermis keratinization (two genes) and they seem to be specific to the EBS-DM phenotype, as they are not significantly differentially expressed between EBS-loc patients and their paired healthy controls. Moreover, In EBS-DM patients, six genes in the epidermis keratinization cluster are keratin filaments or keratin-associated proteins important for the hair shaft or the inner root sheath (Fig. 2). Thus, we hypothesized that the two distinct forms of EBS (EBS-DM and EBS-loc) involve different biological pathways and that the expression profiling differences were mainly between EBS severe phenotype patients and healthy control subjects. We deduce that in skin expressing the keratin mutations of milder EBS disease forms (EBS-loc), gene expression is not significantly changed. Whilst the detailed mechanism underlying this is unclear, we thought that the transcriptomic pattern of milder EBS phenotype patients is quite similar to that of healthy subjects in resting conditions and is particularly influenced by physical stress conditions. This finding is in accordance with a recently published study by Liovic et al. (2009), who analyzed gene expression of cytoskeleton components in cultured EBS-DM cells by microarray profiling and showed that around 20 genes encoding proteins expressed in the keratin-interacting desmosomes and hemidesmosomes, tight junctions and gap junctions are modulated, but none of these genes was significantly changed in EBS-loc patients (Liovic et al. 2009). The authors concluded that only severe mutations could affect intercellular junctions in cultured EBS keratinocytes. In our present work, cell junction components showed no significant alteration in all EBS patients. We hypothesized that the EBS model—immortalized cultured keratinocytes cell lines—used by the authors might have contributed to the apparent difference (Cottage et al. 2001), since we examined the expression profiling directly from the epidermis tissue without exposing it to any type of physiological stress.

When exploring the molecular function of altered expressed genes in our microarrays within the two principal clusters (acid fatty metabolism and epidermis keratinization), we realized that these two pathways are both involved in cornified envelope synthesis. The cornified cell envelope, consisting of a 10-nm thick layer of highly crosslinked insoluble proteins, is formed beneath the plasma membrane in terminally differentiating stratified squamous epithelia and provides a vital physical barrier to these tissues in mammals (Hohl 1990; Matoltsy and Matoltsy 1966; Rice and Green 1977). The physical structure of this epidermal water barrier has been ascribed to sheets of stacked lipid bilayers and many other proteins including cystatin a, desmoplakin, envoplakin, filaggrin, involucrin, keratins, loricrin and the small proline-rich proteins (SPRR) (Steinert and Marekov 1999). Therefore, lipid biosynthesis and SPRR proteins are both implicated in the constitution of water permeability barrier of the stratum corneum. Although Sasaki et al. (1998) showed that abnormalities in keratins 5 and 14 do not alter the patterns of expression of suprabasal keratins and CCE precursor proteins including involucrin, loricrin and small proline-richproteins (SPRs) 1 and 2, by immunohistochemistry and electron microscopy, we observed, in our microarray results, a higher expression of genes of acid fatty metabolism and epidermis keratinization and we hypothesized that it could be a way to stop blister formation and water loss by maintaining cell and tissue integrity in the severe phenotype EBS-DM patients.

Recently, a profiling study in atopic dermatitis patients has revealed a marked differential regulation of genes involved in lipid biosynthesis (Saaf et al. 2008). Numerous other studies of essential fatty acid metabolism and enzymes—including LOX in skin—have been published, notably in psoriasis and other dermatoses including ichtyosis and Darier's disease (Baer et al. 1995; Iversen and Kragballe 2000; Fogh and Kragballe 2000; Jobard et al. 2002; Oxholm et al. 1990). Moreover, It has been demonstrated recently that the sodium 4-phenylbutyrate (4-PBA), a low molecular weight fatty acid (Qi et al. 2004), can act as a therapeutic molecule for EBS (Chamcheu et al. 2011).

It will be interesting to investigate how changes in the keratin cytoskeleton lead to changes in epidermal lipids and cornified envelope proteins in EBS patients. The implication of these two biological processes in EBS should contribute to a better understanding of the metabolic pathways altered in this disease. Future EBS treatments, especially for Dowling-Meara form, might benefit from our microarray findings on fatty acids metabolism.

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Conflict of interest No conflict of interest.

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