TABLE I. Categories of representative proteins detected by proteome analysis

Category	SwissProt accession no.	Protein name	Normal (n = 3)	EAD (n = 8)	IAD (n = 4)	IV (n = 3)	*P* value
1. Inflammation-associated substances	P01876	Ig alpha-1 chain C region	0.29	0.68	0.76	0.76	.666
	P01857	Ig gamma-1 chain C region	0.42	4.68*	1.29	1.57	**.006**
	P01859	Ig gamma-2 chain C region	0.34	0.77	0.85	1.40	.589
	P01860	Ig gamma-3 chain C region	0.40	1.68	0.75	1.27	.082
	P01861	Ig gamma-4 chain C region	0.34	1.74	0.85	1.46	.116
	P01834	Ig kappa chain C region	0.37	2.60	1.18	1.13	.122
	P0CG04	Ig lambda-1 chain C regions	0.28	1.39	0.58	0.85	.236
	P02768	Serum albumin	1.44	16.45*	4.70	4.82	**.039**
2. SC barrier constituents	Q15517	Corneodesmosin	1.06	0.88	1.67	1.27	.299
	Q08554	Desmocollin-1	2.17	2.96	3.54	2.41	.783
	Q02413	Desmoglein-1	5.57	7.34	8.90	6.92	.818
	P15924	Desmoplakin	0.60	0.94	0.77	1.54	.111
	P20930	Filaggrin	2.39	0.11*	0.38	0.25	**.015**
	Q5D862	Filaggrin-2	4.59	1.62	3.21	3.63	.333
3. SC barrier–related enzymes	Q13510	Acid ceramidase	0.71	0.43	1.54	1.96	.052
	P05089	Arginase-1	1.21	0.39	1.05	0.53	**.030**
	Q13867	Bleomycin hydrolase	1.13	0.34	1.01	0.56	**.043**
	P07384	Calpain-1 catalytic subunit	0.21	0.20	0.27	0.16	.881
	P31944	Caspase-14	4.29	4.55	5.56	3.61	.873
	P07339	Cathepsin D	0.78	1.43	0.79	1.88	.732
	Q9Y337	Kallikrein-5	0.16	0.40	0.18	0.19	.620
	P49862	Kallikrein-7	0.32	0.55	0.36	0.64	.571
4. Antimicrobial peptides	P31151	Protein S100-A7	0.33	2.37	0.27	0.93	.149
	P05109	Protein S100-A8	0.96	1.88	2.25	2.98	.666
	P06702	Protein S100-A9	1.10	4.48	1.86	4.58	.316
	P81605	Dermcidin	14.63	4.21	3.37	16.10	.229
5. Sweat-associated substances	P12273	Prolactin-inducible protein (GCDFP15)	16.37	3.65†	12.20	32.91	**.023**

P values were calculated by using ANOVA.
Boldface indicates $P < .05$ by ANOVA test.
*$P < .05$ in normal controls vs patients with EAD with Tukey test.
†$P < .05$ in patients with EAD vs IV with Tukey test.

We categorized the representative substances into 5 groups (Table I). In the samples from patients with AD, considerable amounts of inflammation-associated plasma substances (category 1), such as albumin and Ig gamma-1 chain C region fragments, were included. The doses of these substances were higher in patients with EAD than in patients with IAD. Because the intensities of AD and pruritus were comparable between patients with EAD and IAD (SCORing Atopic Dermatitis, 53.2 ± 14.9 vs 46.3 ± 18.5; visual analog scale of pruritus, 64.8 ± 25.2 vs 69.3 ± 39.1), elevation in the levels of inflammatory substances may be a characteristic of EAD. Even the patients with IV, possessing S1701X, S2554X, or S2889X hetrozygous *FLG* mutation, had higher amounts of inflammatory substances than did healthy controls, suggesting that the barrier perturbation may induce subclinical inflammation.

When we focused on category 2 (SC barrier constituents, Table I), FLG was significantly reduced in patients with EAD and also tended to be low in patients with IAD and IV than in normal healthy controls (Fig 1, *B*). Notably, even in patients with IAD, which is considered to have no severely perturbed barrier function,[6] the amount of FLG was low. Although not statistically significant, FLG-2 was decreased in patients with EAD. Desmosome constituents, including corneodesmosin, desmocollin-1, desmoglein-1, and desmoplakin, were not changed among the 4 groups.

In category 3 (SC barrier–related enzymes), acid ceramidase was increased in patients with IAD and IV, suggesting its possible contribution to ceramide deficiency. Arginase-1, bleomycin hydrolase, calpain-1, caspase-14, and cathepsin D are enzymes to process FLG to natural moisturizing factors. Arginase-1 and bleomycin hydrolase were decreased in patients with EAD, as reported previously.[3] Patients with EAD had increased levels of kallikrein 5 and kallikrein 7, which are involved in corneodesmosome cleavage, protease-activated receptor-2 signal induction, and profilaggrin processing,[7] consistent with previous observations.[8]

Among antimicrobial peptides (category 4), there was a tendency that dermcidin, but not the other proteins, was reduced in patients with EAD and IAD. This suggests that the impaired defense against microbes may be attributable to the reduced production of dermcidin.

Category 5 is represented by prolactin-inducible protein (also known as gross cystic disease fluid protein 15 [GCDFP15]), whose amount was significantly reduced in patients with EAD. Because GCDFP15 is produced by sweat glands,[9] our data presumably reflect the decreased sweating in patients with AD. The reduced production of GCDFP15 was confirmed by immunohistochemical staining of eccrine sweat glands, which showed the positive staining of the epidermal acrosyringium (Fig 1, *C*, left) and the secretary potion of dermal eccrine glands (Fig 1, *C*, right) in the skin of normal healthy persons but not in the skin of patients with AD.

The present proteome study allows us to quantify wide-ranging proteins in SC, and AD is a representative target for this analysis. The clinical accuracy and applicability of this analysis were proven by the reduction in FLG in patients with EAD. Information obtained from this comprehensive study is useful not only for the evaluation of the patient's SC condition but also for the detection of critical proteins involved in the pathogenesis of AD.

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Trends in hospitalizations for food-induced anaphylaxis in US children, 2000-2009

To the Editor:

By most estimates, the US prevalence of food allergies has been increasing. Earlier studies also support a corresponding escalation in health care utilization for food-induced anaphylaxis (FIA), including increased numbers of emergency department (ED) visits and hospitalizations.[1-3] In contrast, a recently published article by our group reported that FIA-related ED visits did not differ statistically for children younger than 18 years between 2001 and 2009.[4] To more fully understand these trends in anaphylaxis care, we used a nationally representative pediatric database to describe the frequency and characteristics of FIA hospitalizations in children over the same time period (2000-2009).

Data were obtained from the Healthcare Cost and Utilization Project Kids' Inpatient Database, the only all-payer pediatric inpatient care database in the United States. The Healthcare Cost and Utilization Project Kids' Inpatient Database consists of a stratified random sample of 12,039,432 inpatient discharges from 27 to 44 states during the 4 periods of our study: 2000, 2003, 2006, and 2009. See this article's Online Repository at www.jacionline.org for a description of data collection and estimation procedures.[5] Our analysis was exempted from human subjects review by our institutional review board.

Patients younger than 18 years were included if 1 of the first 3 diagnosis categories included a relevant diagnosis code for FIA[6]: dermatitis due to food (693.10); anaphylactic shock due to adverse food reaction (995.60-995.69); adverse food reaction, not elsewhere classified (995.70); or other anaphylactic shock (995.00). Sensitivity analyses were performed with (1) more inclusive codes (693.10, 995.00, 995.3 [allergy, unspecified, not elsewhere classified], 995.60-995.69, 995.70); (2) more exclusive codes (693.10, 995.60-995.69, 995.70); and (3) limiting food allergy codes to the first diagnostic category only.

FIA visit rates were calculated and analyzed by age, sex, race (white, black, Asian/Pacific islander, other), Hispanic ethnicity, US region (Northeast, Midwest, South, West), and hospital location/teaching status (rural, urban/teaching, urban/nonteaching). Total costs were estimated by applying the Healthcare Cost and Utilization Project Cost-to-Charge Ratio Files.[5] All charges (amount hospital billed for services) and costs

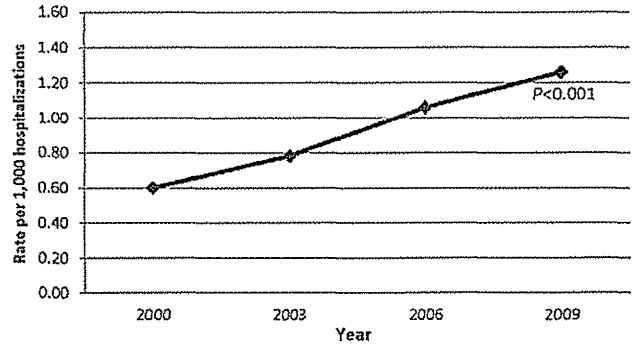

FIG 1. Food-induced anaphylaxis hospitalizations (per 1000 total hospitalizations), by year.

METHODS

Sample collection and storage

The study was approved by the Ethical Committee of Hamamatsu University School of Medicine and performed at the University Hospital of Hamamatsu University School of Medicine. Written informed consent was obtained from all subjects. Human SC samples were obtained from the forearms and upper arms of 3 normal volunteers (3 men; age, 28.3 ± 3.2 years), 8 patients with EAD (5 men and 3 women; age, 28.1 ± 9.1 years), 4 patients with IAD (1 man and 3 women; age, 47.8 ± 6.1 years), and 3 patients with IV (3 men; age, 59.3 ± 22.1 years). Tape stripping was performed at least 24 hours after the last topical application. On sampling, we avoided the skin with severe eczema, moderate to strong lichenification, excoriation, crust, and secondary infection and the skin in joint areas. The SC was obtained by stripping using the Nichiban cellophane tape (organic solvent-stable tape with organic solvent-soluble adhesive; Nichiban, Tokyo, Japan). The tape was applied to the flexor surface of the subjects' forearm and the upper arm. SC samples were obtained from 12 different places using 10 cm length of Nichiban tape. The SC samples on the tape were immediately stored at −20°C until treatment with toluene. When the tape was dipped in 10 mL of toluene, all adhesives were dissolved and any attached SC was suspended. After the insoluble tape backing was removed, the sample was centrifuged at 3000 rpm for 15 minutes. The precipitate was washed with 5 mL of toluene 6 times to remove any residual adhesive. After the toluene treatment, the purified samples were air dried, weighed, and kept at −20°C.

Protein extraction and purification

Dried SC samples were dissolved by 1% SDS lysis buffer (50 mM Tris HCl, pH 6.8, and 1% SDS) in 1.5 mL protein LoBind tube (Eppendorf, Hamburg, Germany). The samples were then homogenated and sonicated for 20 minutes (interval, 30 seconds; sonication, 30 seconds). The suspension was centrifuged at 13,000 rpm for 15 minutes at 4°C. The supernatant was collected and put into a fresh 1.5-mL protein LoBind tube, and 100% acetone was added at 10 times of the sample volume. It was incubated at −80°C overnight and centrifuged at 15,000 rpm for 30 minutes at 4°C. The supernatant was discarded and the pellet was completely dried by using an evaporator for 15 to 30 minutes at room temperature. Dehydrated pellet was dissolved in 10 μL of 7 mol/L urea for 30 minutes at 60°C. Furthermore, 90 μL of 50 mM ammonium bicarbonate was added to the sample solution. Protein concentration was measured by using the Lowry method with DC Protein assay kit (Bio-Rad, Hercules, Calif). All reagents used were Liquid Chromatography - Mass Spectrometry grade.

Protein digestion

Ten microgram proteins were denatured with No-Weight DTT (Thermo Fisher Scientific) for 5 minutes at 95°C and alkylated by iodoacetamide (Thermo Fisher Scientific) for 20 minutes in the dark at room temperature. After 20 minutes, activated trypsin protease, MS grade (Thermo Fisher Scientific), was added to the sample and incubated overnight at 37°C. Trypsin protease reaction was stopped by trifluoroacetic acid (Thermo Fisher Scientific). Peptides were purified with Pierce C18 Spin Columns (Thermo Fisher Scientific), according to the manufacturer's instructions. Purified peptides were dried by using an evaporator at room temperature. Finally, peptides were dissolved in 0.1% formic acid (Thermo Fisher Scientific). All reagents used were LC/MS grade.

Liquid chromatography/MS/MS analysis

Peptides suspension dissolved in 0.1% formic acid was analyzed by using Q Exactive (Thermo Fisher Scientific). Liquid chromatography was carried out using a Thermo Easy-nLC 1000 HPLC equipped using a C-18 column (3 μm particle size, 150 × 0.075 mm internal diameter) (Nikkyo Technos, Co, Ltd, Tokyo, Japan). Eluted peptides were analyzed on Nanospray Flex Ion Source. Buffer A for the pump consisted of 0.1% formic acid in LC/MS-grade water; buffer B for the pump consisted of 0.1% formic acid in LC/MS-grade acetonitrile. Gradient conditions for pump B were as follows: 0% to 35% B from 0 to 120 minutes. A total of 5 μL of the prepared peptides was injected onto the enrichment column for concentration/purification. Flow rates were 300 nL/min. Some parameters in Orbitrap were as follows: spray voltage, 2.0 kV; capillary temperature, 250°C; *m/z* (mass to charge ratio) range (ms), 350 to 1800. AGC ion injection targets for each FTMS scan were 70,000 (60 ms max injection time). AGC ion injection targets for each MS^2 scan were 17,500 (50 ms max ion injection time). Full MS/dd-MS^2 (Top10) was used in this analysis.

Database searching

The raw data was processed using Proteome Discoverer (version 1.4.0.288, Thermo Fischer Scientific). MS/MS spectra were searched with Mascot (version 2.4, Matrix Science, London, United Kingdom) engine against the SwissProt *Homo sapiens* protein sequence database. Peptides were generated from a tryptic digestion with up to 2 missed cleavages, dynamic modifications of methionine oxidation, and a static modification of cysteine carbamidomethylation. Precursor mass tolerance was 10 ppm, and product ions were searched at 0.02-Da tolerances. Peptide spectral matches were validated using percolator based on q values at a 1% false discovery rate.[E1]

Protein selection

For each of the 18 tissue specimen data files (3 normal healthy, 8 EAD, 4 IAD, and 3 IV), Proteome Discover was used to export the list of identified proteins to Excel. For quantification purposes, we utilized the node "The Precursor Ions Area Detector" of Proteome Discoverer, which calculates the area under the curve of each precursor ion using integration. For greater accuracy, it uses an average of the 3 most abundant peptides per protein rather than all peptides per protein to calculate the protein area. The number of peptide spectral matches for each protein in each sample was also used for quantification (spectral counting). We exported a table from Proteome Discoverer that contained SwissProt accession numbers, protein names, the number of peptide spectrum matches, and the protein area for each protein from each sample file. Both the number of spectral counts and the protein area estimates for each protein in each sample were used for further statistical validation. Unmodified/modified and shared (homologous) peptides were also present in the output list from Proteome Discoverer. Dynamic exclusion was set to 10 s.

Statistical analysis

Protein production levels were normalized by glyceraldehyde 3-phosphate dehydrogenase, and these log-transform values were compared between 4 types of groups (healthy, EAD, IAD, and IV) by using ANOVA. Furthermore, multiple comparisons of each of the EAD, IAD, IV groups and healthy controls were conducted by using the Tukey test.

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TABLE E1. Complete list of proteins identified by using liquid chromatography/MS/MS analysis

SwissProt accession no.	Protein name
P62258	14-3-3 protein epsilon
P31947	14-3-3 protein sigma
P63104	14-3-3 protein zeta/delta
P11021	78-kDa glucose-regulated protein
Q13510	Acid ceramidase
P60709	Actin, cytoplasmic 1
O75608	Acyl-protein thioesterase 1
P05141	ADP/ATP translocase 2
P61204	ADP-ribosylation factor 3
Q9UKK9	ADP-sugar pyrophosphatase
Q8TDN7	Alkaline ceramidase 1
P02763	Alpha-1-acid glycoprotein 1
P19652	Alpha-1-acid glycoprotein 2
P01011	Alpha-1-antichymotrypsin
P01009	Alpha-1-antitrypsin
P04217	Alpha-1B-glycoprotein
P01023	Alpha-2-macroglobulin
A8K2U0	Alpha-2-macroglobulin-like protein 1
P12814	Alpha-actinin-1
P49419	Alpha-aminoadipic semialdehyde dehydrogenase
P04745	Alpha-amylase 1
P06733	Alpha-enolase
P06280	Alpha-galactosidase A
P00709	Alpha-lactalbumin
Q16706	Alpha-mannosidase 2
P17050	Alpha-*N*-acetylgalactosaminidase
P04083	Annexin A1
P07355	Annexin A2
P09525	Annexin A4
P08758	Annexin A5
P02647	Apolipoprotein A-I
P02652	Apolipoprotein A-II
P04114	Apolipoprotein B-100
P05090	Apolipoprotein D
O75342	Arachidonate 12-lipoxygenase, 12R-type
O43150	Arf-GAP with SH3 domain, ANK repeat, and PH domain–containing protein 2
P05089	Arginase-1
P54793	Arylsulfatase F
P61769	Beta-2-microglobulin
Q562R1	Beta-actin-like protein 2
P05814	Beta-casein
P16278	Beta-galactosidase
P08236	Beta-glucuronidase
P07686	Beta-hexosaminidase subunit beta
Q13867	Bleomycin hydrolase
Q9NP55	BPI fold-containing family A member 1
Q8TDL5	BPI fold-containing family B member 1
Q9NXV2	BTB/POZ domain–containing protein KCTD5
P12830	Cadherin-1
Q13557	Calcium/calmodulin-dependent protein kinase type II subunit delta
P62158	Calmodulin
P27482	Calmodulin-like protein 3
Q9NZT1	Calmodulin-like protein 5
Q96L46	Calpain small subunit 2
P07384	Calpain-1 catalytic subunit
P00915	Carbonic anhydrase 1
P00918	Carbonic anhydrase 2
P23280	Carbonic anhydrase 6
Q9UI42	Carboxypeptidase A4
P16870	Carboxypeptidase E
P14384	Carboxypeptidase M
P31944	Caspase-14

(Continued)

TABLE E1. *(Continued)*

SwissProt accession no.	Protein name
P04040	Catalase
P49913	Cathelicidin antimicrobial peptide
P07858	Cathepsin B
P07339	Cathepsin D
P08311	Cathepsin G
O60911	Cathepsin L2
P20645	Cation-dependent mannose-6-phosphate receptor
P13987	CD59 glycoprotein
P00450	Ceruloplasmin
P36222	Chitinase-3-like protein 1
Q15782	Chitinase-3-like protein 2
O14493	Claudin-4
P10909	Clusterin
P35606	Coatomer subunit beta
P23528	Cofilin-1
A6NC98	Coiled-coil domain-containing protein 88B
P01024	Complement C3
P0C0L4	Complement C4-A
Q07021	Complement component 1 Q subcomponent-binding protein, mitochondrial
Q15517	Corneodesmosin
Q9BYD5	Cornifelin
P06732	Creatine kinase M-type
P12532	Creatine kinase U-type, mitochondrial
Q9UJ71	C-type lectin domain family 4 member K
P01040	Cystatin-A
P04080	Cystatin-B
Q15828	Cystatin-M
P01036	Cystatin-S
P54108	Cysteine-rich secretory protein 3
Q07065	Cytoskeleton-associated protein 4
Q9UGM3	Deleted in malignant brain tumors 1 protein
Q8NFT8	Delta and Notch-like epidermal growth factor–related receptor
O76062	Delta(14)-sterol reductase
P13716	Delta-aminolevulinic acid dehydratase
P81605	Dermcidin
Q6E0U4	Dermokine
Q08554	Desmocollin-1
Q02487	Desmocollin-2
Q14574	Desmocollin-3
Q02413	Desmoglein-1
P15924	Desmoplakin
P09622	Dihydrolipoyl dehydrogenase, mitochondrial
Q01459	Di-*N*-acetylchitobiase
P53634	Dipeptidyl peptidase 1
Q9UHL4	Dipeptidyl peptidase 2
P27487	Dipeptidyl peptidase 4
P39656	Dolichyl-diphosphooligosaccharide–protein glycosyltransferase 48-kDa subunit
P04843	Dolichyl-diphosphooligosaccharide–protein glycosyltransferase subunit 1
P61803	Dolichyl-diphosphooligosaccharide–protein glycosyltransferase subunit DAD1
P46977	Dolichyl-diphosphooligosaccharide–protein glycosyltransferase subunit STT3A
Q8TEA8	D-tyrosyl-tRNA(Tyr) deacylase 1
P68104	Elongation factor 1-alpha 1
P13639	Elongation factor 2
P61916	Epididymal secretory protein E1
P58107	Epiplakin
P24390	ER lumen protein retaining receptor 1
P56537	Eukaryotic translation initiation factor 6
Q16610	Extracellular matrix protein 1
Q01469	Fatty acid–binding protein, epidermal
Q6ZVX7	F-box only protein 50
P02671	Fibrinogen alpha chain
P02675	Fibrinogen beta chain

(Continued)

TABLE E1. *(Continued)*

SwissProt accession no.	Protein name
P02679	Fibrinogen gamma chain
Q9BYJ0	Fibroblast growth factor–binding protein 2
P02751	Fibronectin
P20930	Filaggrin
Q5D862	Filaggrin-2
P21333	Filamin-A
O75369	Filamin-B
P17931	Galectin-3
Q08380	Galectin-3–binding protein
P47929	Galectin-7
Q92820	Gamma-glutamyl hydrolase
O75223	Gamma-glutamylcyclotransferase
P13284	Gamma-interferon–inducible lysosomal thiol reductase
P17900	Ganglioside GM2 activator
Q96QA5	Gasdermin-A
P06396	Gelsolin
P04062	Glucosylceramidase
Q16769	Glutaminyl-peptide cyclotransferase
P35754	Glutaredoxin-1
P09211	Glutathione S-transferase P
P48637	Glutathione synthetase
P04406	Glyceraldehyde-3-phosphate dehydrogenase
Q7L5L3	Glycerophosphodiester phosphodiesterase domain–containing protein 3
P28799	Granulins
P63244	Guanine nucleotide-binding protein subunit beta-2-like 1
P00739	Haptoglobin-related protein
P08107	Heat shock 70-kDa protein 1A/1B
P11142	Heat shock cognate 71-kDa protein
P04792	Heat shock protein beta-1
P54652	Heat shock–related 70-kDa protein 2
Q9Y5Z4	Heme-binding protein 2
P69905	Hemoglobin subunit alpha
P68871	Hemoglobin subunit beta
P02042	Hemoglobin subunit delta
P02790	Hemopexin
P42357	Histidine ammonia-lyase
P04196	Histidine-rich glycoprotein
Q96KK5	Histone H2A type 1-H
O60814	Histone H2B type 1-K
P62805	Histone H4
P04229	HLA class II histocompatibility antigen, DRB1-1 beta chain
Q86YZ3	Hornerin
Q9BYJ1	Hydroperoxide isomerase ALOXE3
P22304	Iduronate 2-sulfatase
P01876	Ig alpha-1 chain C region
P01877	Ig alpha-2 chain C region
P01857	Ig gamma-1 chain C region
P01859	Ig gamma-2 chain C region
P01860	Ig gamma-3 chain C region
P01861	Ig gamma-4 chain C region
P01825	Ig heavy chain V-II region NEWM
P01772	Ig heavy chain V-III region KOL
P01777	Ig heavy chain V-III region TEI
P01765	Ig heavy chain V-III region TIL
P01834	Ig kappa chain C region
P01619	Ig kappa chain V-III region B6
P01620	Ig kappa chain V-III region SIE
P01625	Ig kappa chain V-IV region Len
P80748	Ig lambda chain V-III region LOI
P01714	Ig lambda chain V-III region SH
P0CG04	Ig lambda-1 chain C regions
P0CG05	Ig lambda-2 chain C regions

(Continued)

TABLE E1. *(Continued)*

SwissProt accession no.	Protein name
P04220	Ig mu heavy chain disease protein
P01591	Immunoglobulin J chain
P12268	Inosine-5′-monophosphate dehydrogenase 2
O14732	Inositol monophosphatase 2
P14735	Insulin-degrading enzyme
Q9NZH8	Interleukin-36 gamma
Q9NZH6	Interleukin-37
Q6ZNF0	Iron/zinc purple acid phosphatase-like protein
P14923	Junction plakoglobin
O43240	Kallikrein-10
Q9UBX7	Kallikrein-11
Q9P0G3	Kallikrein-14
Q9Y337	Kallikrein-5
P49862	Kallikrein-7
O60259	Kallikrein-8
Q9UKQ9	Kallikrein-9
Q15323	Keratin, type I cuticular Ha1
P13645	Keratin, type I cytoskeletal 10
P13646	Keratin, type I cytoskeletal 13
P02533	Keratin, type I cytoskeletal 14
P19012	Keratin, type I cytoskeletal 15
P08779	Keratin, type I cytoskeletal 16
Q04695	Keratin, type I cytoskeletal 17
Q9C075	Keratin, type I cytoskeletal 23
P35527	Keratin, type I cytoskeletal 9
P04264	Keratin, type II cytoskeletal 1
Q7Z794	Keratin, type II cytoskeletal 1b
P35908	Keratin, type II cytoskeletal 2 epidermal
P12035	Keratin, type II cytoskeletal 3
P19013	Keratin, type II cytoskeletal 4
P13647	Keratin, type II cytoskeletal 5
P02538	Keratin, type II cytoskeletal 6A
P48668	Keratin, type II cytoskeletal 6C
P08729	Keratin, type II cytoskeletal 7
Q86Y46	Keratin, type II cytoskeletal 73
O95678	Keratin, type II cytoskeletal 75
Q8N1N4	Keratin, type II cytoskeletal 78
Q6KB66	Keratin, type II cytoskeletal 80
A6NCN2	Keratin-81-like protein KRT121P
Q5T749	Keratinocyte proline-rich protein
P02788	Lactotransferrin
Q659C4	La-related protein 1B
Q9BS40	Latexin
Q9UIQ6	Leucyl-cystinyl aminopeptidase
P30740	Leukocyte elastase inhibitor
P31025	Lipocalin-1
P00338	L-lactate dehydrogenase A chain
Q7Z4W1	L-xylulose reductase
Q9BZG9	Ly-6/neurotoxin-like protein 1
O95274	Ly6/PLAUR domain–containing protein 3
Q6UWN5	Ly6/PLAUR domain–containing protein 5
O95867	Lymphocyte antigen 6 complex locus protein G6c
P10253	Lysosomal alpha-glucosidase
O00754	Lysosomal alpha-mannosidase
P10619	Lysosomal protective protein
P42785	Lysosomal Pro-X carboxypeptidase
Q14108	Lysosome membrane protein 2
P11279	Lysosome-associated membrane glycoprotein 1
P13473	Lysosome-associated membrane glycoprotein 2
P61626	Lysozyme C
P40121	Macrophage-capping protein
P40926	Malate dehydrogenase, mitochondrial

(Continued)

TABLE E1. *(Continued)*

SwissProt accession no.	Protein name
O75556	Mammaglobin-B
P09237	Matrilysin
P08582	Melanotransferrin
P55145	Mesencephalic astrocyte-derived neurotrophic factor
Q99735	Microsomal glutathione S-transferase 2
P26038	Moesin
Q8NHP6	Motile sperm domain–containing protein 2
Q9HC84	Mucin-5B
Q96DR8	Mucin-like protein 1
P24158	Myeloblastin
P05164	Myeloperoxidase
Q7Z406	Myosin-14
P35579	Myosin-9
Q86UT5	Na(+)/H(+) exchange regulatory cofactor NHE-RF4
P15586	*N*-acetylglucosamine-6-sulfatase
Q7L592	NADH dehydrogenase [ubiquinone] complex I, assembly factor 7
P48163	NADP-dependent malic enzyme
Q09666	Neuroblast differentiation-associated protein AHNAK
P59665	Neutrophil defensin 1
P08246	Neutrophil elastase
P80188	Neutrophil gelatinase-associated lipocalin
Q92542	Nicastrin
P51688	*N*-sulphoglucosamine sulphohydrolase
P61970	Nuclear transport factor 2
Q8IXM6	Nurim
Q96FX8	p53 apoptosis effector related to PMP-22
P50897	Palmitoyl-protein thioesterase 1
P62937	Peptidyl-prolyl cis-trans isomerase A
Q06830	Peroxiredoxin-1
P32119	Peroxiredoxin-2
P51659	Peroxisomal multifunctional enzyme type 2
P30086	Phosphatidylethanolamine-binding protein 1
P80108	Phosphatidylinositol-glycan-specific phospholipase D
Q6P1J6	Phospholipase B1, membrane-associated
Q6P4A8	Phospholipase B-like 1
Q9GZP4	PITH domain–containing protein 1
Q13835	Plakophilin-1
P05120	Plasminogen activator inhibitor 2
P13796	Plastin-2
Q15149	Plectin
Q4VXU2	Polyadenylate-binding protein 1-like
P01833	Polymeric immunoglobulin receptor
P0CG48	Polyubiquitin-C
P02545	Prelamin-A/C
Q9UHG3	Prenylcysteine oxidase 1
P07602	Proactivator polypeptide
Q6NUJ1	Proactivator polypeptide-like 1
Q6GTS8	Probable carboxypeptidase PM20D1
O75592	Probable E3 ubiquitin-protein ligase MYCBP2
P09668	Pro-cathepsin H
P07737	Profilin-1
Q9HCJ1	Progressive ankylosis protein homolog
P12273	Prolactin-inducible protein (GCDFP15)
Q99935	Proline-rich protein 1
P15309	Prostatic acid phosphatase
P25786	Proteasome subunit alpha type-1
P25787	Proteasome subunit alpha type-2
P25788	Proteasome subunit alpha type-3
P25789	Proteasome subunit alpha type-4
P28066	Proteasome subunit alpha type-5
P60900	Proteasome subunit alpha type-6
O14818	Proteasome subunit alpha type-7

(Continued)

TABLE E1. *(Continued)*

SwissProt accession no.	Protein name
P20618	Proteasome subunit beta type-1
P49721	Proteasome subunit beta type-2
P49720	Proteasome subunit beta type-3
P28070	Proteasome subunit beta type-4
P28074	Proteasome subunit beta type-5
P28072	Proteasome subunit beta type-6
Q99436	Proteasome subunit beta type-7
P28062	Proteasome subunit beta type-8
O75629	Protein CREG1
O60888	Protein CutA
Q99497	Protein DJ-1
Q9Y4F4	Protein FAM179B
Q8TBQ9	Protein kish-A
Q969L2	Protein MAL2
Q9Y316	Protein MEMO1
P31949	Protein S100-A11
Q9HCY8	Protein S100-A14
Q96FQ6	Protein S100-A16
P31151	Protein S100-A7
P05109	Protein S100-A8
P06702	Protein S100-A9
O43548	Protein-glutamine gamma-glutamyltransferase 5
Q08188	Protein-glutamine gamma-glutamyltransferase E
P22735	Protein-glutamine gamma-glutamyltransferase K
Q04941	Proteolipid protein 2
P00491	Purine nucleoside phosphorylase
Q8NHP8	Putative phospholipase B-like 2
A6NDJ8	Putative Rab-43-like protein ENSP00000330714
Q6ZTY8	Putative uncharacterized protein C12orf63
P14618	Pyruvate kinase PKM
Q86YS3	Rab11 family-interacting protein 4
P20339	Ras-related protein Rab-5A
O95197	Reticulon-3
Q9NQC3	Reticulon-4
Q9HB40	Retinoid-inducible serine carboxypeptidase
Q53RT3	Retroviral-like aspartic protease 1
P34096	Ribonuclease 4
Q9H1E1	Ribonuclease 7
Q5TZA2	Rootletin
P16615	Sarcoplasmic/endoplasmic reticulum calcium ATPase 2
P55000	Secreted Ly-6/uPAR-related protein 1
O95968	Secretoglobin family 1D member 1
O95969	Secretoglobin family 1D member 2
Q9NQ38	Serine protease inhibitor Kazal-type 5
P02787	Serotransferrin
Q8IW75	Serpin A12
P48595	Serpin B10
Q96P63	Serpin B12
Q9UIV8	Serpin B13
P29508	Serpin B3
P48594	Serpin B4
P35237	Serpin B6
O75635	Serpin B7
P50452	Serpin B8
P02768	Serum albumin
Q8NEX9	Short-chain dehydrogenase/reductase family 9C member 7
Q9HAT2	Sialate *O*-acetylesterase
A6NMB1	Sialic acid–binding immunoglobulin-like lectin 16
Q9Y3R4	Sialidase-2
Q5T750	Skin-specific protein 32
P62314	Small nuclear ribonucleoprotein Sm D1
P62318	Small nuclear ribonucleoprotein Sm D3

(Continued)

TABLE E1. *(Continued)*

SwissProt accession no.	Protein name
P08842	Steryl-sulfatase
O00391	Sulfhydryl oxidase 1
P00441	Superoxide dismutase [Cu-Zn]
P04179	Superoxide dismutase [Mn], mitochondrial
Q6UWP8	Suprabasin
O15260	Surfeit locus protein 4
Q16563	Synaptophysin-like protein 1
P10599	Thioredoxin
P04066	Tissue alpha-L-fucosidase
Q9H0E2	Toll-interacting protein
P37837	Transaldolase
Q7Z6R9	Transcription factor AP-2-δ
P29401	Transketolase
P43307	Translocon-associated protein subunit alpha
P51571	Translocon-associated protein subunit delta
Q9UNL2	Translocon-associated protein subunit gamma
Q9HD45	Transmembrane 9 superfamily member 3
P49755	Transmembrane emp24 domain-containing protein 10
Q9Y3B3	Transmembrane emp24 domain-containing protein 7
Q9BVK6	Transmembrane emp24 domain-containing protein 9
Q14956	Transmembrane glycoprotein NMB O
Q9BVC6	Transmembrane protein 109
Q8N2U0	Transmembrane protein 256
Q9BTV4	Transmembrane protein 43
P02766	Transthyretin
P60174	Triosephosphate isomerase
O14773	Tripeptidyl-peptidase 1
P06753	Tropomyosin alpha-3 chain
P68363	Tubulin alpha-1B chain
P68366	Tubulin alpha-4A chain
P04350	Tubulin beta-4A chain
Q93009	Ubiquitin carboxyl-terminal hydrolase 7
Q9NZ01	Very-long-chain enoyl-CoA reductase
Q15836	Vesicle-associated membrane protein 3
O75396	Vesicle-trafficking protein SEC22b
P08670	Vimentin
P18206	Vinculin
P02774	Vitamin D–binding protein
P04004	Vitronectin
P21796	Voltage-dependent anion-selective channel protein 1
Q13698	Voltage-dependent L-type calcium channel subunit alpha-1S
Q86VR7	V-set and immunoglobulin domain-containing protein 10-like
P27449	V-type proton ATPase 16-kDa proteolipid subunit
Q15904	V-type proton ATPase subunit S1
Q86UK7	Zinc finger protein 598
Q3KQV3	Zinc finger protein 792
Q3KNS6	Zinc finger protein 829
P25311	Zinc-alpha-2-glycoprotein
Q96DA0	Zymogen granule protein 16 homolog B

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CONCISE COMMUNICATION

Leukoderma in patients with atopic dermatitis

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ABSTRACT

Atopic dermatitis (AD) is occasionally associated with vitiligo, however, the incidence and conditions of vitiligo or leukoderma, and the characteristics of concurrent AD, remain unclear. We conducted a prospective observational study to investigate the leukoderma-related clinical manifestations and bioparameters of AD. Because vitiligo in AD lesions is occasionally associated with inflammation, we used leukoderma in this study. Enrolled were all AD patients who had been followed up in our AD outpatient clinic and visited within the previous 4 months. During this period, we carefully inspected whether the patients had leukoderma. Eight of 52 patients had leukoderma (15.4%) and were designated as the leukoderma group, and the remaining 44 patients comprised the non-leukoderma group. While the ages were statistically not different between the two groups, female preponderance was significantly observed in the leukoderma group. The leukoderma patients tended to have higher values of SCORAD, CCL17/thymus and activation regulated chemokine and lactate dehydrogenase than the non-leukoderma patients. The leukoderma group was also characterized by a lower frequency of allergic rhinitis and a higher frequency of prurigo lesions. Thus, despite the possession of high AD severity, the leukoderma patients may possibly retain a relatively T-helper 1-skewing state in relation to the development of leukoderma and less association with rhinitis.

Key words: atopic dermatitis, filaggrin, leukoderma, thymus and activation regulated chemokine, vitiligo.

INTRODUCTION

Vitiligo is a disorder in which autoimmune destruction of melanocytes results in white patches of skin. T cells, pro-inflammatory cytokines and autoantibodies can damage melanocytes, and $CD8^+$ cytotoxic lymphocytes (CTL) play a major role.[1] Clinical and extensive genetic studies have shown that vitiligo is associated with other autoimmune diseases, such as thyroid disease,[2] pernicious anemia and diabetes mellitus.

Atopic dermatitis (AD) or atopic diathesis is occasionally associated with vitiligo,[3–7] although its association is less frequent than thyroid disease. Thyroid disorder and AD are more frequent in post-pubertal and pre-pubertal onset vitiligo, respectively.[3] An extremely inflammatory condition of vitiligo, called vitiligo with inflammatory raised border, can arise in AD patients.[5,6] Therefore, it is often difficult to differentiate vitiligo from post-inflammatory depigmentation, and the term leukoderma may be more appropriate to represent the condition. Because both cellular and humoral immune mechanisms are implicated in the development of vitiligo, it is interesting to clarify whether vitiligo/leukoderma occurs more preferentially in T-helper (Th)1- or Th2-skewing condition. AD is well known as Th2 disease, but patients are not homogeneous in severity, serum immunoglobulin (Ig)E level, CCL17/thymus and activation regulated chemokine (TARC) level, filaggrin (FLG) gene loss-of-function mutation[8] and complications. Therefore, it is an issue whether these factors are related to the development of leukodermatous lesions. Here, we conducted a prospective observational study of AD patients to investigate whether the presence of vitiligo/leukoderma is related to the AD-associated clinical manifestations and bioparameters.

METHODS

Atopic dermatitis was diagnosed according to the criteria of Hanifin and Rajka.[9] Enrolled were all AD patients who had been followed up for 2 months to 15 years in our AD outpatient clinic and visited recently between May and August 2014. Prospectively, we carefully inspected whether the patients had leukoderma lesions.

The patients had been characterized by the following clinical and laboratory examinations. The presence of allergic rhinitis and the past history of asthma were recorded. The severity of AD was assessed by severity scoring for AD (SCORAD) and a 100-mm visual analog scale (VAS) was used for pruritus. The presence of pruriginous lesions (subacute, chronic and nodular prurigo) and Dennie–Morgan fold were evaluated. Serum CCL17/TARC, which is strongly associated with severity of AD, was measured by enzyme-linked immunoassay (Special Reference Laboratories, Tokyo, Japan). Blood eosinophil count, serum lactate dehydrogenase (LDH) and total serum IgE were routinely measured. Specific IgE to *Dermatophagoides*

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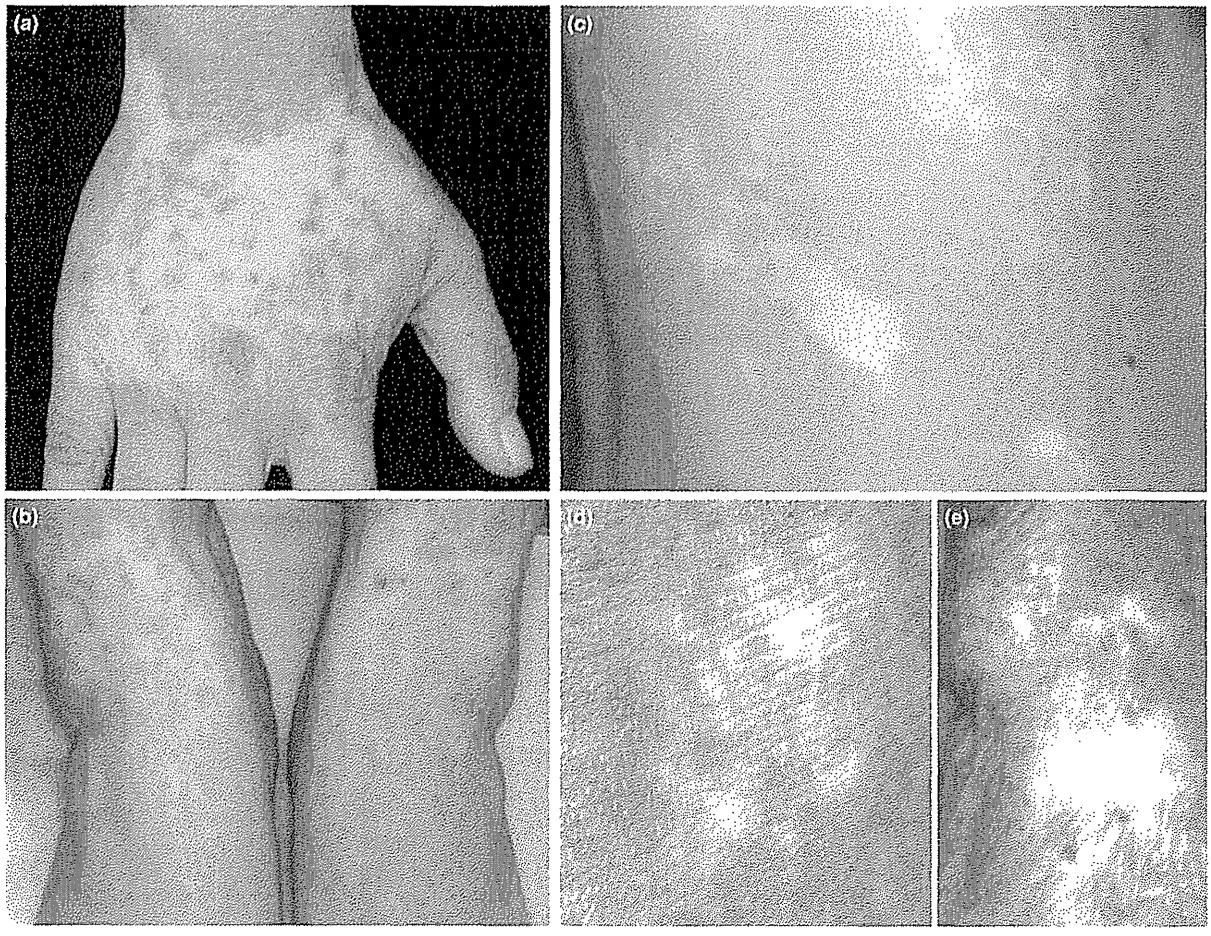

Figure 1. Clinical appearance of leukoderma in atopic dermatitis patients. (a,b) Vitiligo-like lesions on the dorsum of the right hand and the extensor surface of the bilateral feet and shins in a 33-year-old woman. She also had leukoderma lesions on the forehead. (c) Vitiligo-like lesions on the right lateral aspect of the trunk in a 29-year-old woman. (d) Leukoderma lesions on the flexor surfaces of the thighs and calves in a 40-year-old woman. (e) Leukoderma lesions on the distal part of the forearms in a 63-year-old woman.

pteronyssinus (DP) and *Dermatophagoides farinae* (DF) were classified into seven classes (class 0–6).

Genomic DNA was extracted from peripheral blood mononuclear cells of AD patients for detection of *FLG* mutation by polymerase chain reaction (PCR). Genotyping for the eight types of *FLG* (R501X, 3321delA, S1695X, Q1701X, S2554X, S2889X, S3296X and K4022X)[8] was performed using PCR and restriction enzyme digest analysis.[10,11] The clinical and immunological study of AD patients (no. 25–166, 27 September 2013) and the analysis of *FLG* mutation in AD patients (no. 25–241, 20 December 2013) were approved by the ethical committee of Hamamatsu University School of Medicine. Patients gave written informed consent.

Mann–Whitney *U*-test or Fisher's exact test were applied to determine differences. $P < 0.05$ was defined statistically significance.

RESULTS

During 4 months, a total of 52 AD patients were surveyed for the presence of leukoderma. They were aged 7–63 years and composed of 31 men and 21 women. Eight patients (15.4%) had leukoderma, whose sites included hands ($n = 4$), forearms ($n = 2$), lower legs ($n = 2$), face ($n = 2$), feet ($n = 1$) and trunk ($n = 1$) (Fig. 1). The numbers of leukoderma sites were: five or more sites, one case; four sites, two cases; two sites, four cases; and one site, one case. We tentatively divided depigmented patches into vitiligo-like and leukodermatous lesions. A vitiligo-like lesion was defined as a well-demarcated, sharply white patch, while a leukodermatous lesion exhibited an ill-defined white patch with eczema. Two of the eight cases had vitiligo-like lesions. One case exhibited discrete white patches on the bilateral hands (Fig. 1a), the extensor surfaces of ankles

Table 1. Patients' backgrounds and frequencies of various clinical symptoms and bioparameters

	Leukoderma (+) group	Leukoderma (–) group	*P*
Age (years)	40.0 ± 13.1 (*n* = 8)	31.8 ± 14.0 (*n* = 44)	0.144
Sex (M : F)	1:7	15:7	0.0051
Disease duration (years)	34.1 ± 5.5 (*n* = 8)	25.1 ± 14.7 (*n* = 44)	0.122
SCORAD	65.4 ± 18.0 (*n* = 8)	52.5 ± 18.4 (*n* = 42)	0.09
VAS of pruritus	70.4 ± 29.0 (*n* = 7)	62.9 ± 21.4 (*n* = 41)	0.242
LDH (IU/L)	329.1 ± 110.9 (*n* = 8)	260.7 ± 107.9 (*n* = 41)	0.062
Eosinophils (%)	17.1 ± 13.1 (*n* = 8)	10.3 ± 7.2 (*n* = 42)	0.19
Serum TARC (pg/mL)	9961.4 ± 12,050.3 (*n* = 7)	3420.6 ± 7557.3 (*n* = 44)	0.087
Serum IgE (IU/L)	13 365 ± 15 197 (*n* = 8)	7436.8 ± 10 507.7 (*n* = 44)	0.127
Allergic rhinitis	2/8 (25%)	28/42 (66.7%)	0.0469
Asthma	2/8 (25%)	11/42 (26.2%)	1
Dennie–Morgan fold	1/8 (12.5%)	8/43 (28.6%)	1
Prurigo	5/8 (62.5%)	10/42 (23.8%)	0.0426
FLG mutation	1/6 (16.7%)	5/38 (13.1%)	1

Mann–Whitney *U*-test was used to determine the differences between the means. Fisher's exact test was applied to determine differences between the frequencies. IgE, immunoglobulin E; LDH, lactate dehydrogenase; TARC, thymus and activation regulated chemokine; VAS, visual analog scale.

(Fig. 1b) and the forehead to frontal scalp. Another case showed irregularly shaped white patches on the trunk (Fig. 1c). Although they strikingly resembled vitiligo, inflammatory red papules were scattered in and around the white patches, and the overlying hairs were not whitish. In the other six cases, the leukoderma lesions were more remarkably intermingled with atopic eczema, and the margin of white patches was not highly discrete (Fig. 1d,e).

The patients were divided into the leukoderma and non-leukoderma groups. The patients' backgrounds are summarized in Table 1. While the ages of the two groups were statistically not different, female preponderance was significantly observed in the leukoderma group. The disease duration tended to be longer in the leukoderma patients, but not statistically significant. The leukoderma patients had significantly higher eosinophil counts and tended to have higher values of SCORAD, CCL17/TARC and LDH than the non-leukoderma patients. Thus, the severity of AD seems to be higher in the leukoderma group. However, there were no significant correlations between the leukoderma number and SCORAD, VAS, IgE, TARC or eosinophil percentage. Although asthma was comparably seen in the two groups, the frequency of allergic rhinitis was significantly lower in the leukoderma group. The leukoderma patients had prurigo lesions at a significantly higher frequency. There were no significant differences between the two groups in VAS of pruritus, total serum IgE levels, IgE levels specific to DP and DF, or *FLG* mutations. None of the leukoderma patients had present or past history of thyroid disease.

DISCUSSION

White patches in AD have been reported as vitiligo, and our present study follows the line of such previous studies. The higher levels of SCORAD, eosinophil counts, CCL17/TARC and LDH observed in the leukoderma group indicate that patients with severe AD are prone to developing leukoderma. Although not statistically significant, the disease duration was longer in the leukoderma patients, suggesting that leukoderma may develop with persistent eczema. Despite possession of the Th2-biased high AD severity, the leukoderma group had less frequent allergic rhinitis, suggesting that certain Th2-preponderant conditions are rather suppressed in the leukoderma group. In general, CTL attack melanocytes in the pathogenesis of vitiligo.[1] Given that CTL are activated by Th1 cytokines and depressed by Th2 cytokines, the relatively Th1-skewing state in relation to the lack of allergic rhinitis may lead to the feasible development of leukoderma.

The higher incidence of leukoderma was remarkably observed in female patients. AD can be divided into the extrinsic and intrinsic types, and the intrinsic type is characterized by female predominance, lower serum IgE levels and relative Th1 activation.[11] The relationship between the leukoderma group and the intrinsic type of AD is not clearly demonstrated in this study, but the immunological condition of intrinsic AD may promote the formation of leukoderma.

The leukoderma patients had prurigo lesions more frequently than did the non-leukoderma group. Although the mechanism underlying this association is currently unknown, prurigo[12] and vitiligo[13] share skin infiltration of Th17 cells with each other. Th17 cells may be involved in the pathogenesis of both lesions as well as AD.[14]

Vitiligo/leukoderma and AD are mediated by Th1/cytotoxic T (Tc)1 cells and Th2 cells, respectively. It is considered that the comorbidity of AD and leukoderma takes place as a result of the preponderant local immune reaction to Th1/Tc1 and/or Th17 cells. In this concept, the simultaneous occurrence of alopecia areata with AD provides the same ambivalent condition.[15] The mechanism underlying the concurrence of local Th1/Tc1 disease in systemic Th2 disease is an issue to be clarified in the future.

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CONFLICT OF INTEREST: None declared.

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Towards the next stage of novel biomarker discussion in COPD: Tekizai-Tekisho

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Biomarkers predicting clinical outcomes reported in COPD but more data needed for their clinical use http://ow.ly/qm2CZ

Forced expiratory volume in 1 s (FEV1) as a degree of airflow limit2ation has traditionally been regarded as a crucial predictor of mortality in patients with chronic obstructive pulmonary disease (COPD). However, the situation has changed over the past 10 years. Various systemic clinical outcome measurements, such as exercise capacity, dyspnoea, health status, *etc.*, have been found to be associated with mortality [1, 2]. Thus, combined multidimensional disease severity grading protocols have been proposed. To date, the BODE index (body mass index, dyspnoea, exercise capacity and airflow limitation) has led the field as a predictor of disease [3].

Being liberated from the "FEV1 supremacy", COPD has been increasingly recognised as a heterogeneous disease. "Systemic COPD" is one of phenotypes associated with elevated markers of systemic inflammation and a high prevalence of comorbidities. The systemic inflammatory phenotype yields poor clinical outcomes with increased mortality and exacerbation frequency [4]. Systemic inflammation in COPD cannot be explained simply by a "spill-over" phenomenon of inflammatory mediators from the lungs [5]. Other factors such as bronchial colonisation, obesity, hypoxia and comorbidities are involved. Therefore, a single marker does not seem to be sufficient in assessing systemic effects of COPD, and a combination of multiple markers may have advantages.

Given our recent understanding of this situation, the BODE index may not sufficiently represent factors related to such systemic inflammation. Cross-sectionally, associations between the BODE index and systemic inflammatory biomarkers were not strong [6]. Similarly, from the longitudinal point of view, some serum biomarkers have an additive predictive value of mortality with the BODE index [7, 8]. In addition, regarding comorbidities, DIVO *et al.* [9] reported that the qualitative risk stratification comorbidity tool was predictive of risk of death which complimented the BODE index.

In this issue of the *European Respiratory Journal*, STOLZ *et al.* [10] have focused on the role of the adrenomedullin (ADM) fragment, proADM, compared to the BODE index in the prediction of clinical outcomes in a multicentre, prospective, observational study. ADM is a ubiquitous peptide synthesised in a number of tissues and cell types and has a range of biological actions including vasodilation, regulation of hormone secretion, cell growth, natriuresis and antimicrobial effects. ADM has increasingly received focus as a potential novel cardiac biomarker in clinical practice, in addition to the conventionally used natriuretic peptides. The present study was performed after a preceding single centre observational study by these authors showed that plasma proADM concentration on admission to hospital for acute exacerbation of COPD was independently predictive of 2-year all-cause mortality [11].

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STOLZ *et al.* [10] then discovered that proADM itself was most significantly predictive of mortality in patients with stable COPD over other tested blood biomarkers, similarly to the BODE index, and when combined with BODE the predictive power was improved compared to BODE alone (referred to as refined BODE or "BODE-A" index) [10]. In addition, beneficially, proADM plus BOD (body mass index, dyspnoea and airflow limitation), "BOD-A" index, was more predictive of mortality than the original BODE index. This is good news because the 6-min walking test (6MWT) is time-consuming and difficult to perform, especially in the primary clinic. In turn, this has led to attempts to find other possible multidimensional disease severity staging tests that exclude exercise capacity, such as the ADO index (age, dyspnoea and airflow limitation) or DOSE index (dyspnoea, airflow limitation, smoking status and exacerbation frequency). Although the 6MWT is performed for multiple reasons and cannot be substituted by biomarkers alone in predicting mortality, the use of biomarkers is so straightforward that proADM and its related multidimensional indices are promising outcome measurements in COPD.

Why is proADM such a good biomarker in COPD, when it was originally known as a cardiac biomarker? Perhaps we should not be surprised, considering that cardiovascular disease is common and is one of main causes of death in COPD. STOLZ *et al.* [10] have summarised proposed mechanisms in their article, whereby ADM is produced in response to inflammation, infection and hypoxia, which may reflect complex pathogenesis of COPD. Furthermore, in a cross-sectional study [12], increased plasma proADM concentrations were moderately correlated with lower peak oxygen uptake on progressive cycle ergometry in chronic lung diseases (46% COPD) independently of FEV_1, and, in a subgroup analysis, independently of left ventricular ejection fraction. This may indicate that increased plasma proADM partly acts beyond pulmonary or left ventricular functions. Of course, pulmonary hypertension (PH) is an important complication of COPD. However, abnormal haemodynamic vascular responses to exercise or right ventricular dysfunction and remodelling were recently reported to be present even without PH [13, 14], indicating that heart–lung interactions and right ventricular function are noticeable exercise-limiting factors in COPD. Furthermore, an enlarged pulmonary artery on computed tomography, due to several pathological processes besides resting PH, was associated with severe exacerbations of COPD [15]. Thus, ADM may reflect overall impaired cardiopulmonary circulation in COPD, a point which needs further study.

A limitation of the study by STOLZ *et al.* [10] is that the duration of 2 years is relatively short compared to similar studies investigating the association with mortality. In fact, 1- and 2-year mortality rates were low at 4.7% and 7.8%, respectively, indicating that it remains to be elucidated to what extent intervention targeting this biomarker would be beneficial to patients. Although cancer is usually one of the major causes of death in addition to COPD and cardiovascular disease, the study duration in this case may have been too short to permit deaths due to cancer. Therefore, the significance of biomarkers may be different from other studies, and further investigation under various study settings would be needed to confirm future clinical applicability. For example, the recent Spanish COCOMICS study has evaluated and compared the abilities of different multidimensional indices to predict mortality from 6 months to 10 years [16].

Many systemic biomarkers predicting clinical outcomes have recently been reported in COPD. These are mostly based on analysing their relationship with mortality or exacerbations, which will be useful in risk stratification. These studies usually need a large sample size and a long-term follow-up period, and are challenging and exciting for researchers. We should welcome and recognise their efforts when a novel marker is discovered.

However, for clinical applicability of a disease severity marker, clinicians need more information in addition to its predictive property of mortality. This situation might be similar to that in which various novel multidimensional disease indices have been reported for COPD since publication of the original BODE index [17]. Without more robust studies to reveal their properties or characteristics, clinicians might feel rather confused about which, when and how one index should be used, or how it would impact on daily practice. For example, we previously reported that peak oxygen uptake on progressive cycle ergometry and the modified Medical Research Council (mMRC) dyspnoea scale were highly predictive of mortality in COPD [1, 2]. However, endurance procedures would be better at evaluating the effects of bronchodilators on exercise capacity than peak oxygen uptake [18], and, for long-term follow-up of dyspnoea, other multidimensional dyspnoea measurements rather than mMRC (with an approximate five-point scale) would be appropriate [19, 20]. These indicate that, in spite of their excellent predictive properties, their evaluative properties were not as useful, depending on the situation.

A Japanese proverb states: "Tekizai-Tekisho" (teki-zai means most appropriate resource, and teki-sho means most appropriate place). The combination of these two phrases translates to "to place the right resource (human) in the right place" in English. However, a clear understanding is indispensable to such placement. Therefore, when measuring biomarkers in various clinical settings, it is important to be familiar with their properties or characteristics and confirm their appropriateness to the setting in question. For

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clinical researchers, discovery of a novel biomarker is just the start, not the ultimate objective! Considerably more information regarding specificity, responsiveness, stability, superiority and possible beneficial combinations with other markers should be accumulated for clinical applicability going forward. It is not until then that clinicians will gain straightforward access to "a blood biomarker" in assessing COPD.

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proliferation and an enhancement of stimuli responses. Metabolic process genes are highly enriched in both hyper- and hypomethylated groups indicates that the shifting of metabolic processes might be important in skin ageing and may represent a fruitful area for further inquiry.

We also observed a locus of a keratin gene cluster is hypermethylated in ageing skin, and this has been partially cross-validated by a human counterpart study that both Krt1 and Krt16 are hypermethylated in skin ageing (20). However, omics-level study including MeDIP-chip is easily biased, the careful confirmation work is essential if any findings are interested to pursue further. Skin fibroblasts can be induced towards pluripotent stem cells by a combination of transcription factors, and it is essentially an epigenetic reprogramming process (30). Therefore, there is a likelihood that modulation of epigenetics could be an innovative anti-skin-ageing strategy in the future.

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XX conceived this project, HQ performed the research, and XX wrote the paper. This work was supported by Yale University. We appreciate LEN for reviewing/editing the manuscript.

Conflict of interests

The authors have declared no conflicting interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Genome-wide distribution of skin age-induced DNA methylation patterns.

Figure S2. Gene clusters change their methylation status with skin ageing.

Table S1. Age-induced hypermethylated promoters.

Table S2. Age-induced hypomethylated promoters.

Data S1. Materials and methods.

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Letter to the Editor

Topical E6005, a novel phosphodiesterase 4 inhibitor, attenuates spontaneous itch-related responses in mice with chronic atopy-like dermatitis

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Abstract: Atopic dermatitis is a chronic inflammatory cutaneous disease with difficult-to-treat pruritus. Although phosphodiesterase (PDE) 4 inhibitors have an anti-inflammatory effect on inflammatory skin diseases, such as atopic dermatitis, their acute antipruritic activities remain unclear. Therefore, in this study, we examined whether E6005, a novel PDE4 inhibitor, has antipruritic activity in dermatitis, using a mouse model of atopic dermatitis (NC/Nga mice). A single topical application of E6005 inhibited spontaneous hind-paw scratching, an itch-associated response and spontaneous activity of the cutaneous nerve in mice with chronic dermatitis. The cutaneous concentration of cAMP was significantly decreased in mice with chronic dermatitis, and this decrease was reversed by topical E6005 application. These results suggest that E6005 increases the cutaneous concentration of cAMP to relieve dermatitis-associated itching. Thus, E6005 may be a useful therapy for pruritic dermatitis such as atopic dermatitis.

Key words: atopic dermatitis – cAMP – itch – mice – phosphodiesterase

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Background

Atopic dermatitis is a chronic inflammatory skin disease characterized by severe difficult-to-treat pruritus (1). Itching in atopic dermatitis may be mediated by manifold mediators, including interleukin-31 (2), thymic stromal lymphopoietin (3), tryptase (4), leukotriene B_4 (5) and sphingosylphosphorylcholine (6) and may not be fully relieved by selective receptor antagonists. Therefore, effective antipruritic medicines with novel mechanisms of action are required for the treatment of atopic dermatitis. Phosphodiesterase 4 (PDE4), which catalyses the conversion of cAMP to 5′-AMP, plays a critical role in the pathogenesis of inflammatory disorders. PDE4 inhibitors exert anti-inflammatory influences in inflammatory diseases (7–11), and topical application of PDE4 inhibitors exerts anti-inflammatory effects in atopic dermatitis (12). However, it is unclear whether PDE4 inhibitors have acute antipruritic activity in atopic dermatitis.

Questions addressed

E6005 was recently developed as a topically available selective PDE4 inhibitor and has anti-inflammatory and antipruritic activities in allergic contact dermatitis in mice (13). However, it remains unclear whether E6005 has antipruritic activity in chronic dermatitis. Therefore, we examined the acute effects of topical E6005 application on spontaneous itching behaviour, spontaneous activity of the cutaneous nerve and the cutaneous concentration of cAMP in mice with chronic dermatitis.

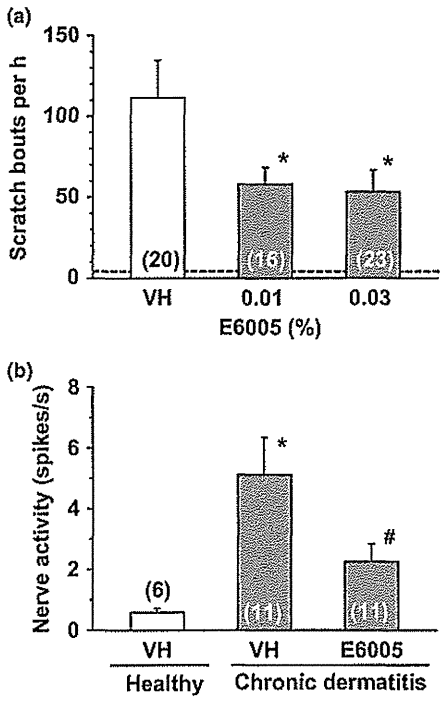

Figure 1. Effects of the PDE4 inhibitor E6005 on spontaneous scratching and cutaneous nerve firing in mice with dermatitis. (a) Effects of E6005 on spontaneous scratching. E6005 and vehicle (VH) were applied topically to the hair-clipped rostra back 1 h before the start of observation. The number of scratching episodes was counted for 1 h. A dashed line indicates the number of spontaneous scratching in healthy controls ($n = 8$). $^*P < 0.05$ vs VH (Holm-Šidák test). (b) Effect of E6005 on spontaneous activity of the cutaneous nerve. The firing of the cutaneous nerve innervating the rostral back including the hair-clipped region was electrophysiologically recorded for 30 min. E6005 (0.03%) and VH were applied to the hair-clipped skin 1 h before recording. $^*P < 0.05$ vs VH in healthy mice, $^\#P < 0.05$ vs VH in mice with dermatitis (Holm–Šidák test). Values represent mean ± SEM. The number in parentheses indicates the number of animals used.

Experimental design

Experimental procedures are detailed in Data S1. Briefly, Male NC/Nga mice with chronic dermatitis (14) and healthy NC/Nga mice were used. E6005, dissolved in an acetone–ethanol mixture, was applied to the hair-clipped rostral back. Observation of hind-paw scratching and electrophysiological recording of the cutaneous nerve branch were conducted according to our previous study (15). The effects of topical application of E6005 were evaluated 1 h after application, because topical application of the vehicle elicited hind-paw scratching for 40 min (Figure S1). The cutaneous concentration of cAMP was measured by enzyme immunoassay.

Results

Although mice with chronic dermatitis scratched throughout the rostral part of the body and head with the hind paws, in this study, we counted scratching of the hair-clipped skin. Healthy mice scratched only several times per hour, while mice with chronic dermatitis frequently scratched the hair-clipped skin (Fig. 1a). A single topical application of E6005 significantly inhibited spontaneous scratching during 1–2 h after application in mice with chronic dermatitis; the inhibition was partial and similar between 0.01% and 0.03% (Fig. 1a).

Spontaneous activity of the cutaneous nerve innervating the rostral back was recorded from 1 to 1.5 h after topical application. The spontaneous activity was as low as 0.6 spikes/s in healthy mice and markedly increased in mice with chronic dermatitis (Fig. 1b). Topical application of 0.03% E6005 to the rostral back significantly inhibited the increased activity of the cutaneous nerve in mice with chronic dermatitis (Fig. 1b). E6005 (0.03%) did not affect nerve activity evoked by punctuate stimulation with a von Frey filament (Figure S2).

The cutaneous concentration of cAMP was significantly decreased in mice with chronic dermatitis (Fig. 2). The decreased concentration of cAMP was significantly reversed 1 h after topical application of 0.03% E6005 in these mice (Fig. 2).

Conclusions

We found that a single topical application of the novel PDE4 inhibitor E6005 inhibited spontaneous hind-paw scratching in mice with chronic dermatitis, suggesting acute antipruritic activity. A single topical application of E6005 reduced the increased activity of the cutaneous branch of the nerve innervating the lesioned skin. These results suggest that E6005 acts locally on the lesioned skin to inhibit the generation of itch

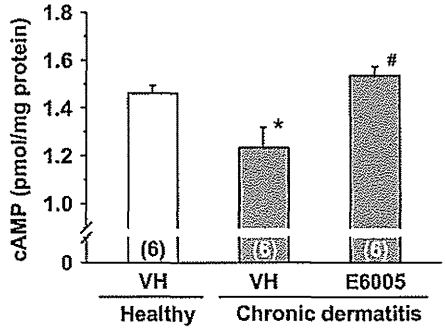

Figure 2. Effect of E6005 on cAMP concentration in the healthy and chronic lesioned skin of mice. E6005 (0.03%) and vehicle (VH) were applied topically to the rostral part of the back 1 h before skin sampling. The concentration of cAMP was measured using a cAMP enzyme immunoassay kit. Values represent mean ± SEM ($n = 6$). $^*P < 0.05$ vs VH in healthy skin, $^\#P < 0.05$ vs VH in dermatitis skin (Holm-Šidák test). The number in parentheses indicates the number of animals used.

signals. Topical application of E6005 did not affect mechanical stimulation-evoked nerve activity, suggesting that the inhibitory effects of E6005 are not due to nervous damage or local anaesthetic action.

The cutaneous concentration of cAMP was decreased in mice with dermatitis, and topical E6005 application reversed the decreased concentration. PDE4 is expressed in the keratinocytes (16). Thus, it is possible that the activation of PDE4 (a decrease in the cAMP concentration) in the epidermal keratinocytes is responsible for chronic dermatitis-associated itching and that the inhibition of activated PDE4 relieves the itching. The mechanisms of the cAMP-mediated antipruritic effect remain unclear. Protein kinase A inactivates 5-lipoxygenase through the phosphorylation of its Ser^{523} residue, leading to a decrease in leukotriene B_4 production (17). Leukotriene B_4 is a potent itch mediator (18) acting on the BLT1 leukotriene B_4 receptor in primary afferents (19). Leukotriene B_4 produced in keratinocytes is involved in spontaneous scratching in mice with dermatitis (5) and proteinase-activated receptor 2-mediated scratching (20). Thus, it is conceivable that an increase in cAMP concentration by E6005 results in the inhibition of increased production of leukotriene B_4 in keratinocytes in dermatitis.

Tryptase release from mast cells is increased in the lesioned skin of patients with atopic dermatitis (21) and NC mice with chronic dermatitis (4). PDE4 inhibitors suppress the degranulation of mast cells through the increase in intra-cellular cAMP (22). Inflammatory cells including T cells are increased in the skin of NC mice with dermatitis (23), T-cell cytokines including interleukin-31 are associated with scratching (2,24), and PDE4 inhibitors suppress the activation and cytokine release of these cells (25). Thus, the anti-inflammatory actions may also contribute to the antipruritic effects of E6005. Sensory C-fibres play an important role in spontaneous scratching in NC mice with dermatitis (26). The sensitivity of C-fibres to heat and chemical stimuli are increased or not affected by cAMP (27), and protein kinase A activation reduces TRPV1 channel desensitization (28). Thus, the direct action of E6005 on primary afferents may not play a role in its antipruritic effect.

The present study showed that E6005, a novel PDE4 inhibitor, has acute antipruritic activity in chronic dermatitis. Thus, E6005 is expected to be useful for the treatment of pruritic chronic dermatitis including atopic dermatitis.

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Author contributions

TA and YK designed experiments, and TA and TY performed experiments. TA and YK wrote the manuscript.

Conflict of interests

The authors do not report any conflict of interest relevant to this study.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Methods.

Figure S1. Hind-paw scratching following topical application of vehicle for E6005 in mice.

Figure S2. E6005 does not inhibit cutaneous nerve activity evoked by mechanical stimulation in mice.

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Letter to the Editor

Genotype analysis in Hungarian patients with multiple primary melanoma

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Abstract: Multiple primary melanoma patients (MPMps) have better prognosis and are more prone to genetic predisposition than single melanoma patients. We aimed to compare genetic background (*CDKN2A, CDK4, MITF, MC1R*) of 43 Hungarian