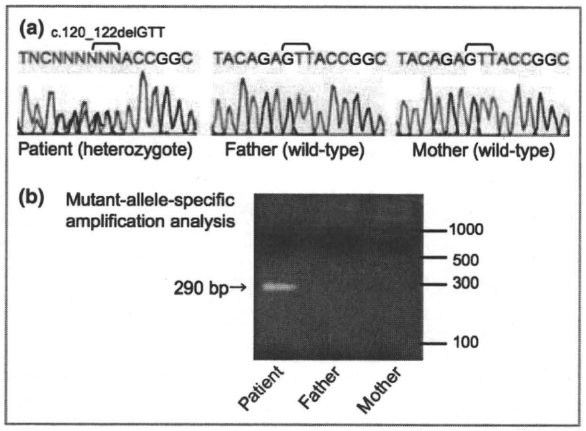

Fig 2. A heterozygous in-frame three-nucleotide deletion mutation c.120_122delGTT in *ATP2A2* was detected. (a) Direct sequencing of *ATP2A2* exon 2 polymerase chain reaction products by a reverse primer revealed that the patient was heterozygous for the three-nucleotide deletion mutation c.120_122delGTT. This mutation was not detected in genomic DNA samples from the patient's parents. (b) Mutant allele-specific amplification analysis shows the amplification band from the mutant allele as a 290-bp fragment only from the DNA sample of the patient, confirming the mutation c.120_122delGTT in the patient.

hand, mutational analysis showed no mutation in *ATP2A2* and genotyping and linkage analysis results revealed no linkage evidence to the locus including *ATP2A2* in a large Chinese family with AKV.[19] Thus, AKV might be a genetically heterogeneous disorder. In any case, further accumulation of cases with molecular genetic assessment is needed to improve understanding of the pathogenesis of variant phenotypes of DD such as CDD and AKV.

Acknowledgments

We thank Akari Nagasaki, MS, for her technical assistance. This work was supported in part by grants from the Ministry of Education, Science, and Culture of Japan (Kiban B 20390304) to M.A.

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Epidermolysis Bullosa in Japan

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KEYWORDS

• Epidermolysis bullosa • Japan • Epidemiology • DebRa

Epidermolysis bullosa (EB) is a group of hereditary disorders characterized by mechanical stress-induced blistering of the skin and mucous membranes.[1] EB is generally classified into the 3 main subtypes of EB simplex (EBS), junctional EB (JEB), and dystrophic EB (DEB), depending on the level of skin cleavage.[1] According to the National EB Registry (USA), the prevalence of EB in the Unites States in terms of cases per million population is estimated to be 8.22 (EBS, 4.60; JEB, 0.44; dominant DEB [DDEB], 0.99; recessive DEB [RDEB], 0.92).[1] The prevalence of EB in Japan in terms of cases per million is estimated to be 4.03 to 5.16 (EBS, 1.54; JEB, 0.34; DDEB, 1.02; RDEB, 1.60), based on data from the Japanese Study Group for Rare Intractable Skin Diseases in 1994.[2] However, the precise disease frequency of EB in Japan is still controversial.

Genetic studies of Japanese patients have revealed specific mutations and distinct tendencies in the genes responsible for the 3 EB subtypes. For example, the proportion of Japanese patients with EBS with *KRT5* mutations is 3 times higher than those with *KRT14*,[3] whereas outside of Japan, mutations in these 2 genes have been reported as equally prevalent. In the *LAMB3* gene, which is associated with JEB, the recurrent mutations R42X and R635X are more common among Caucasians than among ethnic Japanese.[4] The mutations 5818delC, 6573+1G>C, E2857X, and Q2827X have been regarded as recurrent *COL7A1* mutations associated with DEB in Japan.[5,6]

The medical expenses at the hospital for patients with JEB and DEB are covered under the public expenditure system, and 333 JEB and DEB patients in Japan are certified to receive medical care. However, the expense of the dressings and bandages, which is necessary for EB care, is not covered, and the patients have to purchase all that they need. Guidelines for the diagnosis and treatment of EB have been drafted by the Japanese Study Group for Rare Intractable Skin Diseases.[7] In March 2008, the Dystrophic Epidermolysis Bullosa Research Association (DebRA) of Japan was founded (http://www.ne.jp/asahi/eb-japan/com/english1.html), and more than 50 patients with EB and their families have been registered.

The environment surrounding patients with EB has been slowly improving, but support for such patients is still not sufficient (eg, the government finally begins moves to cover part of their dressing costs). EB patients, dermatologists, dermatologic researchers, and the government must interact more closely to improve the quality of life for these patients.

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Dermatol Clin 28 (2010) 431–432
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RESEARCH ARTICLE

Human Mutation

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Prevalent *LIPH* Founder Mutations Lead to Loss of P2Y5 Activation Ability of PA-PLA$_1\alpha$ in Autosomal Recessive Hypotrichosis

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Communicated by David S. Rosenblatt

Received 8 January 2010; accepted revised manuscript 22 February 2010.
Published online 8 March 2010 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/humu.21235

ABSTRACT: Autosomal recessive hypotrichosis (ARH) is characterized by sparse hair on the scalp without other abnormalities. Three genes, *DSG4*, *LIPH*, and *LPAR6* (*P2RY5*), have been reported to underlie ARH. We performed a mutation search for the three candidate genes in five independent Japanese ARH families and identified two *LIPH* mutations: c.736T>A (p.Cys246Ser) in all five families, and c.742C>A (p.His248Asn) in four of the five families. Out of 200 unrelated control alleles, we detected c.736T>A in three alleles and c.742C>A in one allele. Haplotype analysis revealed each of the two mutant alleles is derived from a respective founder. These results suggest the *LIPH* mutations are prevalent founder mutations for ARH in the Japanese population. *LIPH* encodes PA-PLA$_1\alpha$ (LIPH), a membrane-associated phosphatidic acid-preferring phospholipase A$_1\alpha$. Two residues, altered by these mutations, are conserved among PA-PLA$_1\alpha$ of diverse species. Cys^{246} forms intramolecular disulfide bonds on the lid domain, a crucial structure for substrate recognition, and His^{248} is one amino acid of the catalytic triad. Both p.Cys246Ser- and p.His248Asn-PA-PLA$_1\alpha$ mutants showed complete abolition of hydrolytic activity and had no P2Y5 activation ability. These results suggest defective activation of P2Y5 due to reduced 2-acyl lysophosphatidic acid production by the mutant PA-PLA$_1\alpha$ is involved in the pathogenesis of ARH.
Hum Mutat 31:602–610, 2010. © 2010 Wiley-Liss, Inc.

KEY WORDS: LIPH; Lysophosphatidic Acid; Phosphatidic Acid; Lid Domain; Catalytic Triad; LAH2; LAH

Introduction

Autosomal recessive hypotrichosis (ARH; MIM#s 607892, 607903, 611452) is a rare form of alopecia characterized by sparse hair on the scalp, sparse to absent eyebrows and eyelashes, and sparse axillary and body hair. Wali et al. [2007] noted clinical similarities among three genetically distinct forms of hypotrichosis, localized autosomal recessive hypotrichosis (LAH), and proposed that the forms mapped to chromosome 18q12.1, 3q27.2, and 13q14.11–q21.32 are designated as LAH1, LAH2, and LAH3, respectively. Recently, causative genes for all three forms were identified. Mutations in the desmoglein-4 gene (*DSG4*; MIM# 607892) lead to LAH1 [Kljuic et al., 2003; Rafique et al., 2003]. Mutations in *LIPH* (MIM# 607365), which encodes membrane-associated phosphatidic acid-preferring phospholipase A$_1\alpha$ (PA-PLA$_1\alpha$[LIPH]), underlie LAH2 [Ali et al., 2007; Kazantseva et al., 2006]. Most recently, Pasternack et al. [2008] and Shimomura et al. [2008] reported that mutations in the lysophosphatidic acid receptor 6 gene *LPAR6* (*P2RY5*; MIM# 609239) caused LAH3.

In this study, we searched for mutations in the *DSG4*, *LIPH*, and *LPAR6* genes in five unrelated Japanese families with ARH. Surprisingly, we found two prevalent missense mutations in the *LIPH* gene in all of the families. Furthermore, one mutation c.736T>A (p.Cys246Ser) was found in all five families, and the other mutation c.742C>A (p.His248Asn) was detected in four of the five families. We clarified that these two mutations are strong founder mutations in *LIPH* in the Japanese population. In addition, we evaluated the enzyme activity of mutant PA-PLA$_1\alpha$ derived from the two mutant alleles. We also analyzed the abilities of the mutant PA-PLA$_1\alpha$ to activate lysophosphatidic acid receptor 6 (P2Y5), to clarify the pathogenetic pathway of ARH.

Materials and Methods

Subjects

Five unrelated nonconsanguineous Japanese families A, B, C, D, and E (Fig. 1) with ARH were seen in our hospital or referred to us for the past 5 years. Families A, C, and D were from Hokkaido, the northern most major island of Japan. Families B and E were from western and central Japan, respectively. The medical ethics committee of Hokkaido University approved all the described studies. The study was conducted according to the Declaration of Helsinki Principles. The patients gave written informed consent.

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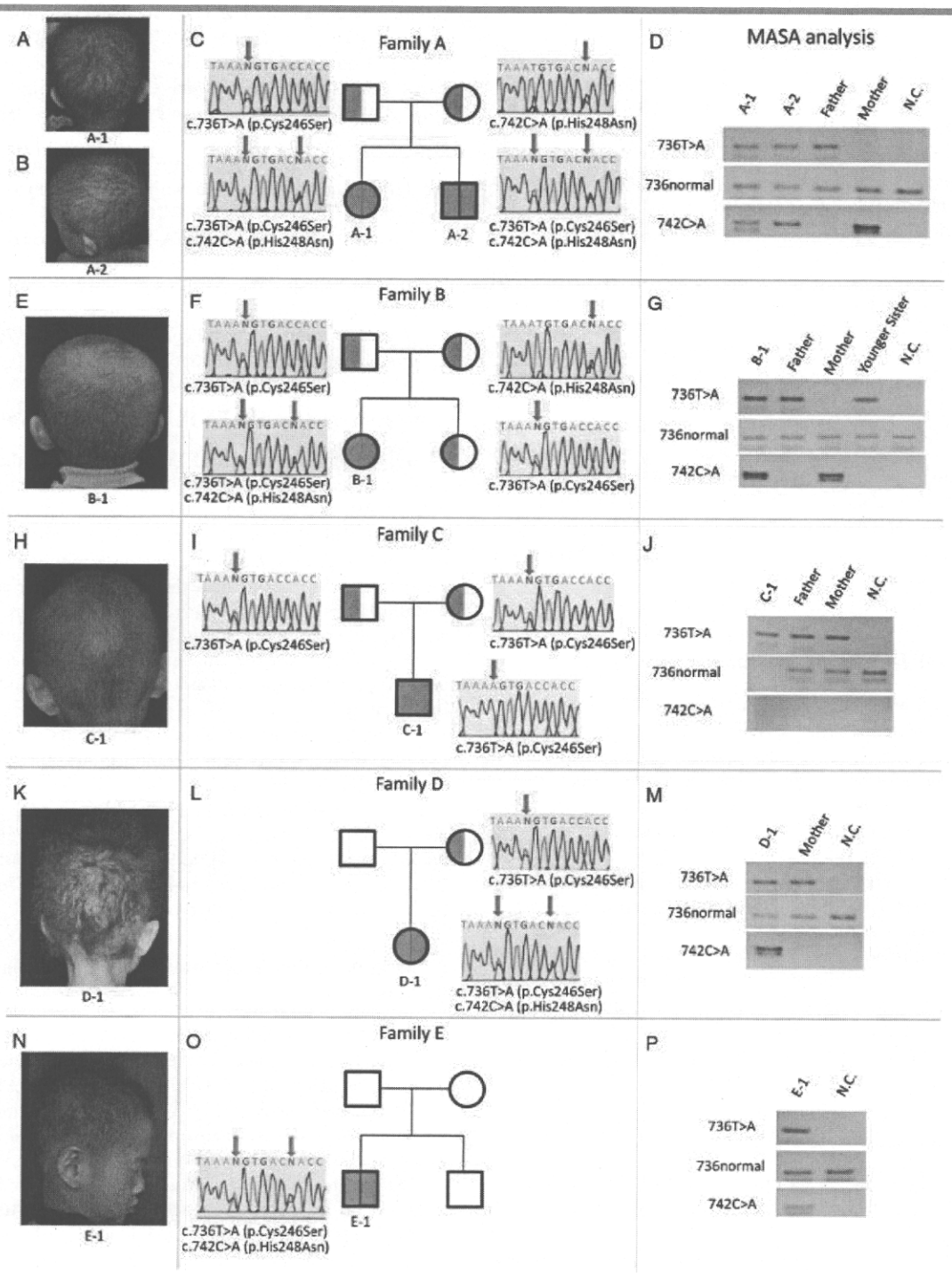


Figure 1. Clinical features of five Japanese families with ARH and identification of mutations in the *LIPH* gene. **A, B, E, H, K, N:** All the affected individuals have features of ARH, which is characterized by sparse hair on the scalp and slightly sparse to absent eyebrows and eyelashes. **C, F, I, L, O:** Pedigrees of the families. Family A (C), Family B (F), Family C (I), Family D (L), and Family E (O) are consistent with autosomal recessive inheritance. Direct sequencing of the *LIPH* gene revealed that patients A-1, A-2, B-1, D-1, and E-1 had compound heterozygous missense mutations involving c.736T>A and c.742C>A, whereas patient C-1 had a homozygous c.736T>A missense mutation. **D, G, J, M, P:** Mutant-allele-specific amplification (MASA) analysis. (Upper) With c.736T>A mutant allele-specific primers, the amplification bands from the c.736T>A mutant alleles are detected by direct sequencing as 301 bp fragments only in the patients and their family members who had the c.736T>A missense mutation, confirming the presence of the mutation. (Middle) With c.736 wild-type allele-specific primers, no PCR product was detected in patient C-1, who was homozygous for c.736T>A. PCR products from the other patients who were compound heterozygous for the two missense mutations c.736T>A and c.742C>A, from unaffected family members and from the normal control (N.C.) were amplified by wild-type allele-specific amplification. (Lower) With c.742C>A mutant-allele-specific primers, the amplification bands from the c.742C>A mutant alleles were detected as 297 bp fragments only in the PCR products from the DNA samples of the patients and their family members who had the c.742C>A missense mutation, confirming the presence of the mutation.

Mutation Detection

DSG4, *LIPH*, and *LPAR6* mutation search was performed as previously reported [Moss et al., 2004; Pasternack et al., 2008; Shimomura et al., 2008, 2009b]. Briefly, genomic DNA (gDNA) isolated from peripheral blood was subjected to polymerase chain reaction (PCR) amplification, followed by direct automated sequencing using an ABI PRISM 3100 genetic analyzer (Advanced Biotechnologies, Columbia, MD), and verification of the mutations by mutant-allele-specific amplification (MASA) analysis.

Oligonucleotide primers were designed using the Website program (www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). The entire coding regions of *DSG4*, *LIPH*, and *LPAR6*, including the exon/intron boundaries, were sequenced using gDNA samples from patients and their family members, after fully informed consent. For normal controls, 100 healthy unrelated Japanese individuals (200 normal alleles) were studied.

The complementary DNA (cDNA) nucleotides and the amino acids of the protein were numbered based on the previous sequence information (GenBank accession number, *DSG4*; AY177664.1, *LIPH*; AY093498.1, *LPAR6*; AF000546.1) [Jin et al., 2002; Whittock and Bower, 2003]. Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, according to journal guidelines (www.hgvs.org/mutnomen). The initiation codon is codon 1.

Mutant Allele-Specific Amplification Analysis

For verification of the mutation, using PCR products as a template, mutant allele specific amplification analysis was performed with mutant allele-specific primers carrying the substitution of a base at the 3′-end [Hasegawa et al., 1995; Xu et al., 2003], as follows: c.736T>A mutant allele-specific forward primer, 5′-CCAAGGATTTCAGTATTTTAAAA-3′; c.736 normal allele-specific forward primer, 5′-CCAAGGATTTCAGTATTT-TAAAT-3′; c.742C>A mutant allele-specific forward primer, 5′-GGATTTCAGTATTTTAAATGTGACA-3′; reverse primer, 5′-GTGCCCAGCAGAAAAACAAG-3′.

PCR conditions were as follows: 94°C for 5 min, followed by 35 cycles at 94°C for 1 min, 60°C (for c.736T>A mutant amplification) or 64°C (for c.742C>A mutant amplification) for 1 min, and extension at 72°C for 7 min. Only 301- and 297-bp fragments derived from the mutant alleles were amplified with these primers and the PCR condition, respectively.

Haplotype Analysis

To determine whether the mutations c.736T>A and c.742C>A are founder mutations, we performed haplotype analysis. We constructed linkage disequilibrium (LD) blocks containing the *LIPH* gene using genotype data from the HapMap database (International HapMap Consortium, 2005). The haplotype structure with its tag-single nuclotide polymorphisms (SNPs) was determined using Haploview [Barrett et al., 2005]. We genotyped 10 tag-SNPs using the ABI PRISM 3100 genetic analyzer (Advanced Biotechnologies). Oligonucleotide primers were designed using the website program (www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi).

Construction of Mutated *LIPH* Gene Expression Vectors

Normal human full-length *LIPH* cDNA was amplified by reverse transcription-PCR using human colon-derived total RNA [Sonoda et al., 2002]. The DNA fragment covering the coding region of PA-PLA$_1\alpha$ (EcoRI–EcoRI fragment) was subcloned into the EcoRI site of pCAGGS mammalian expression vector (kindly donated by Dr. Junichi Miyazaki, Osaka University) [Hiramatsu et al., 2003]. Short *LIPH* fragments (64 bp) (c.695–758) including either the c.736T>A or the c.742C>A mutation were synthesized by IDT Inc. (Coralville, IA). pCAGGS vector including the rest of the *LIPH* gene was amplified with specific primers as follows: forward (5′-CCTGTACCTGTCTTCCCTGAG-3′) and reverse (5′-CAGGTTGATCCAATCCTCCA-3′). PCR was carried out using KOD-Plus-Ver.2 (Toyobo, Osaka, Japan) according to the instructions. Finally, the synthesized mutated DNA fragments were ligated with the amplified pCAGGS vector including the *LIPH* gene without 64 bp oligonucleotide (c.695–758) using a Ligation-Convenience Kit (Nippon Gene Co., Tokyo, Japan).

Expression of Mutated PA-PLA$_1\alpha$ in HEK293 Cells

To investigate the molecular defects underlying the mutations that were identified in this study, we synthesized p.Cys246Ser or p.His248Asn mutations in PA-PLA$_1\alpha$ expression constructs and compared mutant protein expression with wild-type (WT) and p.Ser154Ala PA-PLA$_1\alpha$ protein. Previously, Sonoda et al. [2002] reported that Ser^{154} was the active catalytic residue and that the p.Ser154Ala mutant PA-PLA$_1\alpha$ had complete loss of enzyme activity, although the amount of p.Ser154Ala mutant protein expressed was almost the same as that of WT protein. Thus, we used the p.Ser154Ala mutant as a loss-of-function mutant control in this study.

HEK293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with antibiotics and 10% fetal bovine serum under an atmosphere of 5% CO_2 at 37°C. The resulting cDNAs were used to transfect HEK293 cells using LipofectAMINE 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. HEK293 cells were transfected with WT, p.Ser154Ala (control loss-of-function mutant) [Sonoda et al., 2002], p.Cys246Ser or p.His248Asn PA-PLA$_1\alpha$.

Preparation of Cell Supernatants and Lysates and Western Blotting

HEK293 cells transfected with pCAGGS vector were maintained for an additional 24 hr after the medium was changed to serum-free medium ExCell302 (JRH Biosciences, Lenexa, KS). After 24 hr of incubation, the media were collected and precipitated with trichloroacetic acid. Precipitated protein was collected by centrifugation at 15,000 × *g* for 20 min, followed by washing with acetone twice; then, the pellet was redissolved in sodium dodecyl sulfate (SDS) sample buffer A (62.5 mM Tris-HCl [pH 6.8], 10% Glycerol, 2% SDS, 5% 2-mercaptoethanol (2ME), 10 μg/mL phenylmethylsulphonyl fluoride [PMSF]) and boiled for 5 min. HEK293 cells were harvested 48 hr after transfection and SDS sample buffer B (62.5 mM Tris-HCl [pH 6.8], 4 M Urea, 10% Glycerol, 2% SDS, 5% 2ME, 10 μg/mL PMSF) was added directly to the cell pellet. The pellet was then sonicated and boiled for 5 min.

These protein samples of cell supernatants and lysates were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membrane. The nitrocellulose membrane was blocked with Tris-buffered saline containing 5% (w/v) skimmed milk and 0.05% (v/v) Tween 20, incubated with anti-PA-PLA$_1\alpha$ monoclonal antibody [Sonoda et al., 2002], and then treated with antirat IgG antibody conjugated with horseradish peroxidase. Proteins bound to the antibodies were

visualized with an enhanced chemiluminescence kit (ECL, Amersham Biosciences, Piscataway, NJ) by LAS4000 Luminescent Image Analyzer (Fujifilm, Tokyo, Japan) [Sonoda et al., 2002].

PA-PLA$_1\alpha$ Enzyme Activity Assay

PA-PLA$_1\alpha$ produces 2-acyl lysophosphatidic acid (LPA) and free fatty acid (FFA) concurrently from phosphatidic acid (PA) [Sonoda et al., 2002]. In the present study, the hydrolysis activity was determined measuring oleic acids, which are concurrently produced from dioleoyl PA by PA-PLA$_1\alpha$. We added the supernatant from HEK293 cells transfected with WT, p.Ser154Ala, p.Cys246Ser, or p.His248Asn PA-PLA$_1\alpha$ to the medium including 100 μM PA. After 12 hr incubation at 37°C, the amount of oleic acids was measured with NEFA C-Test Wako test kit (Wako Chemicals Co., Osaka, Japan).

P2Y5 Activation Ability Assay

We cotransfected alkaline-phosphatase-tagged transforming growth factor-α (AP-TGFα) (kindly provided by Dr. Higashiyama, Ehime University, Japan) [Tokumaru et al., 2000], recombinant P2Y5 and WT, p.Ser154Ala, p.Cys246Ser, or p.His248Asn PA-PLA$_1\alpha$ to HEK293 cells, and we quantified free AP-TGFα induced by a disintegrin and metalloprotease (ADAM) in the HEK293 cells to examine the P2Y5 activation ability of LPA produced by mutant PA-PLA$_1\alpha$. Cells were cultured in 100 μL of serum-free medium Opti-MEM (Gibco BRL, Grand Island, NY) in individual wells of a 96-well plate. After 24 hr of incubation, 80 μL of the conditioned medium in each well was transferred and AP activities in both the conditioned media and the transfected cells were measured using *p*-nitrophenyl phosphate (*p*-NPP). In the case of phorbol ester, 12-O-tetradecanoylphorbol-13-acetate (TPA)-stimulation, the transfected cells were treated with 100 nM 1 h before medium transfer. The AP reaction was performed in *p*-NPP buffer (5 mM *p*-NPP, 20 mM Tris-HCl (pH 9.5), 20 mM NaCl, and 5 mM $MgCl_2$) at 37°C for 1 hr and the increases in the reaction product, *p*-nitrophenol, were quantified by monitoring absorbance at 405 nm with VersaMax microplate reader (Molecular Devices, Sunnyvale, CA). The amount of AP-TGFα released was expressed as a ratio of AP activity in the conditioned media to total AP activity in each well.

Results

Clinical Findings

All six affected individuals in the five unrelated Japanese families showed features typical of ARH (Fig. 1A, B, E, H, K, and N). The patients were less than 10 years of age at the time of the study. Affected individuals had tightly curled hair, which grew slowly and stopped growing after a few inches. Their eyebrows and eyelashes were a little sparse to absent. Nails, teeth, sweating, and hearing were normal in all the affected individuals. Heterozygous carriers had normal hair. The pedigrees of all the families were consistent with autosomal recessive inheritance (Fig. 1C, F, I, L, and O).

Mutation Detection

Direct sequencing analysis of exons and intron–exon boundaries of *LIPH* revealed that affected members of Families A, B, D, and E were compound heterozygous for the two missense mutations c.736T>A (p.Cys246Ser) and c.742C>A (p.His248Asn) (Fig. 1C, F, I, L, O). The affected individual in Family C was homozygous for c.736T>A. All the parents whose DNA was available for mutation search were heterozygous carriers of one of the two mutations (Fig. 1C, F, I, L, and O). We confirmed these *LIPH* mutations by MASA analysis (Fig. 1D, G, J, M, and P). Both amino acid residues altered by the two missense mutations were highly conserved among diverse species (Fig. 2A). One of the mutations was found in 4/200 normal unrelated alleles (100 healthy Japanese individuals) by direct sequence analysis (minor allele frequency, c.736T>A, 0.015 (3/200); c. 742C>A, 0.005 (1/200); combined genotype 0.02 (4/200)), although there was no control individual who had compound heterozygous or homozygous mutations (data not shown). No other pathogenic mutation was found in the entire exon or intron/exon borders of the *DSG4*, *LIPH* or *LPAR6* gene.

Haplotype Aanalysis

The haplotype block structure containing the *LIPH* gene was constructed using genotype data from the HapMap database (Fig. 3B). The haplotype block was represented by five haplotypes with >1% frequency (Fig. 3C). The haplotype of the chromosome containing the *LIPH* c.736T>A mutation was found to have resulted from parent-to-child transmission in all five families (Table 1). The chromosome containing the *LIPH* c.736T>A mutation had haplotype I (ATCAACCGGA), which is seen in 37.8% of the Han Chinese and ethnic Japanese populations. Likewise, we determined the haplotype of the chromosome containing the *LIPH* c.742C>A mutation in four families (A, B, D, E). The chromosome containing the *LIPH* c.742C>A mutation had haplotype III (GCTCGTGAGG), which is seen in 28.9%. Thus, these missense mutations c.736T>A (p.Cys246Ser) and c.742C>A (p.His248Asn) in Japanese patients appear to represent founder effects in this island nation.

Expression of PA-PLA$_1\alpha$ in Mammalian Cells

Immunoblot analysis revealed that transfection of p.Cys246Ser and p.His248Asn mutant constructs into HEK293 cells resulted in the secretion of 55-kDa mutant PA-PLA$_1\alpha$ at a level similar to that of the WT and of the p.Ser154Ala mutant (Fig. 4A). In addition, the same amounts of mutant PA-PLA$_1\alpha$ proteins were also recovered from the cell lysate. These results indicated that there was no significant difference in protein yield between WT and mutant PA-PLA$_1\alpha$.

Analysis of PA-PLA$_1\alpha$ Hydrolytic Activity

The hydrolysis activity was determined measuring FFA which are concurrently produced from PA by PA-PLA$_1\alpha$. The quantities of FFA produced by the p.Cys246Ser and p.His248Asn mutant *LIPH* constructs were similar to those by the mock and p.Ser154Ala mutant constructs, suggesting that the p.Cys246Ser and p.His248Asn mutant PA-PLA$_1\alpha$ had no hydrolytic activity (Fig. 4B).

P2Y5 Activation Ability of PA-PLA$_1\alpha$ Mutants

In this study, we cotransfected AP-TGFα, recombinant P2Y5 and WT, p.Ser154Ala, p.Cys246Ser, or p.His248Asn PA-PLA$_1\alpha$ constructs to HEK293 cells. To examine the P2Y5 activation potency of mutant PA-PLA$_1\alpha$, AP-TGFα release into conditioned media via ADAM, which was triggered by activation of P2Y5, was

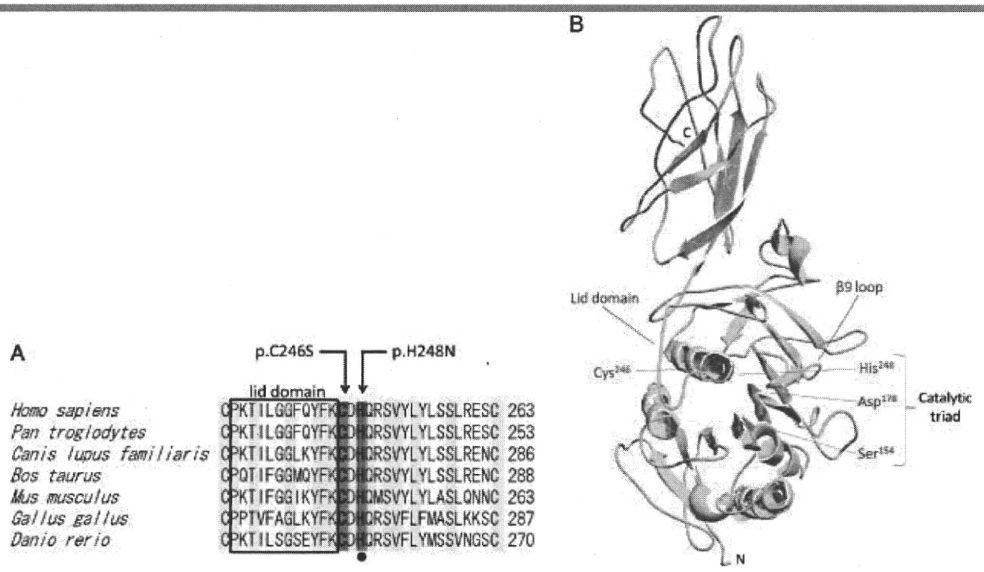
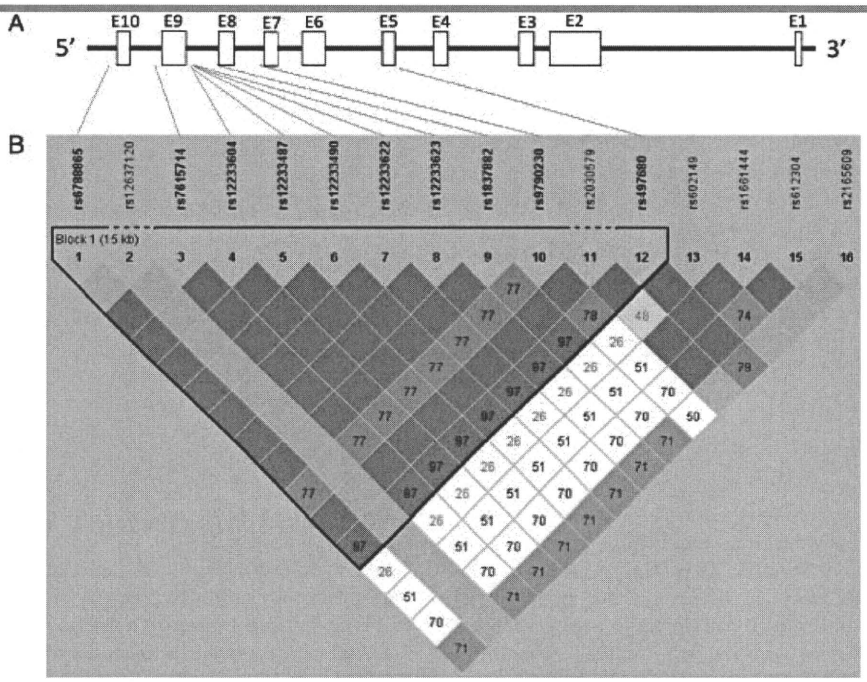


Figure 2. Conservation of the mutated residues and the three-dimensional protein structure around the mutation sites. **A**: Multiple amino acid sequence alignments of PA-PLA$_1\alpha$ of diverse species. Amino acid residues Cys^{246} and His^{248} altered by the present two mutations are highly conserved among PA-PLA$_1\alpha$ of diverse species. Amino acid residues that are conserved between the seven species are shown in yellow. The 12 residues that comprise the lid domain are surrounded by a black rectangle. One of the amino acids of the catalytic triad, His^{248}, is marked with a black dot. Cys^{246} and His^{248} are in red and indicated by arrows. **B**: The three-dimensional-structure model of PA-PLA$_1\alpha$ protein. Cys^{246} and His^{248} residues are in red. Lid domain and β9loop are in green. Catalytic triad consists of Ser^{154} (purple), Asp^{178} (purple) and His^{248}. Cys^{246} forms intramolecular disulfide bonds on the lid domain.



C

Haplotype	SNP											Haplotype Frequency
	rs6788865	rs7615714	rs12233604	rs12233487	rs12233490	rs12233622	rs12233623	rs1837882	rs9790230	rs497680		
I	A	T	C	A	A	C	C	G	G	A		0.378
II	A	T	C	A	A	C	C	G	A	A		0.294
III	G	C	T	C	G	T	G	A	G	G		0.289
IV	G	C	T	C	G	T	G	A	A	G		0.022
V	A	T	C	A	A	C	C	G	G	G		0.011

Figure 3. The linkage disequilibrium (LD) block and the haplotype structure around *LIPH* in Han Chinese and ethnic Japanese populations. *LIPH* structure (**A**) and the LD block within *LIPH* (**B**) were evaluated using genotype data from the HapMap database. **C**: The haplotype structure with 10 tag-SNPs was determined using Haploview.

Table 1. Identified Haplotype with the *LIPH* c.736T>A and c.742C>A Mutation

Family	Mutation	rs6788865	rs7615714	rs12233604	rs12233487	rs12233490	rs12233622	rs12233623	rs1837882	rs9790230	rs497680	Haplotype
A	c.736T>A	A/G	T/C	C/T	A/C	A/G	C/T	C/G	G/A	G/G	A/G	I/III
	c.742C>A	A/G	T/C	C/T	A/C	A/G	C/T	C/G	G/A	G/G	A/G	I/III
B	c.736T>A	A	T	C	A	A	C	C	G	G	A	I
	c.742C>A	G	C	T	C	G	T	G	A	G	G	III
C (homozygote)	c.736T>A	A	T	C	A	A	C	C	G	G	A	I
D	c.736T>A	A	T	C	A	A	C	C	G	G	A	I
	c.742C>A	G	C	T	C	G	T	G	A	G	G	III
E	c.736T>A	A/G	T/C	C/T	A/C	A/G	C/T	C/G	G/A	G/G	A/G	I/III
	c.742C>A	A/G	T/C	C/T	A/C	A/G	C/T	C/G	G/A	G/G	A/G	I/III

Nucleotide numbering starts at +1 corresponding to the A of the ATG initiation codon in the reference sequence AY093498.1 (www.hgvs.org/mutnomen). SNP, single-nucleotide polymorphism.

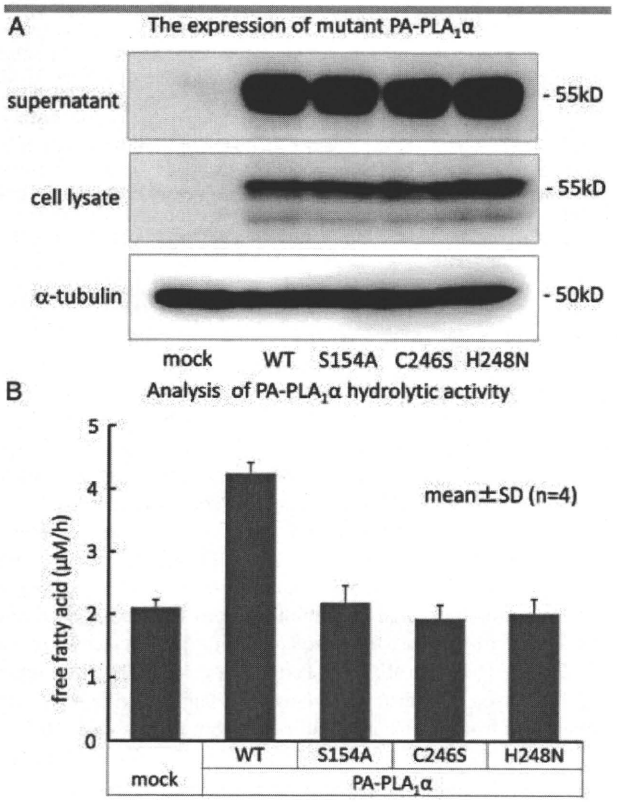


Figure 4. Expression of PA-PLA$_1\alpha$ in HEK293 cells and its hydrolytic activity. **A:** Expression of mutant PA-PLA$_1\alpha$ in HEK293 cells. HEK293 cells were transfected with wild-type (WT), p.Ser154Ala (S154A), p.Cys246Ser (C246S), and p.His248Asn (H248N) *LIPH* cDNA, and the expression level of PA-PLA$_1\alpha$ protein derived from the constructs in cell culture supernatant (upper panel) and cells (middle panel) were evaluated by Western blot. There were no significant differences in PA-PLA$_1\alpha$ protein expression levels among cells trasfected with WT, S154A, C246S, and H248N. α-tubulin expression was used as a standard to assess the total amount of proteins from cell lysate loaded on the gel (lower panel). **B:** Because PA-PLA$_1\alpha$ hydrolyzes the free fatty acid (FFA) from PA, we monitored the levels FFA to determine whether there is a difference in the PA-PLA$_1\alpha$ hydrolytic activity among WT and the three mutants of PA-PLA$_1\alpha$. After 12-hr incubation of the supernatant from HEK293 cells expressing WT, S154A, C246S, or H248N PA-PLA$_1\alpha$, with a medium including 100 μM PA, the levels of FFA hydrolyzed by C246S and H248N mutant PA-PLA$_1\alpha$ were significantly lower than that by WT PA-PLA$_1\alpha$ and similar to those produced by supernatant from HEK293 cells transfected with control S154A mutant and an empty vector (mock).

quantified using *p*-NPP as a substrate for AP. The free AP-TGFα from the P2Y5 mock transfected (P2Y5-) cells transfected with the WT form of PA-PLA$_1\alpha$ was more abundant than that from the P2Y5− cells transfected with empty vector, which indicated that the HEK293 cells had the ability to shed TGFα mediated by intrinsic LPA receptor at some level (Fig. 5A). AP-TGFα release from P2Y5 positive (P2Y5+) cells expressing the WT PA-PLA$_1\alpha$ was remarkably increased compared with mock or mutant PA-PLA$_1\alpha$. There were no significant differences between the data obtained with cells expressing the mutants and the empty vector (Fig. 5A). All the cells expressing AP-TGFα responded equally to TPA, confirming that expression of P2Y5 and PA-PLA$_1\alpha$ did not affect PKC-dependent AP-TGFα release (Fig. 5B). These data clearly indicated that these mutations resulted in the loss of P2Y5 activation activity of PA-PLA$_1\alpha$.

Discussion

The human *LIPH* gene encodes PA-PLA$_1\alpha$, which is a member of the membrane-associated phosphatidic acid-preferring phospholipase A$_1\alpha$ [Hiramatsu et al., 2003; Jin et al., 2002; Sonoda et al., 2002]. Similar to other phospholipase A$_1$, PA-PLA$_1\alpha$ has N-terminal domains that are essential for catalytic activity. Three amino acid residues, Ser^{154}, Asp^{178}, and His^{248}, which form the putative catalytic triad, are located in the N-terminal domains [Aoki et al., 2007; Jin et al., 2002; Kubiak et al., 2001; Sonoda et al., 2002] (Fig. 2B). PA-PLA$_1\alpha$ has a β9 loop (the 13 amino acids from p.206 to 218) and a short lid domain (the 12 amino acids from p.234 to245), each of which is considered a crucial structure for substrate recognition [Aoki et al., 2007; Carriere et al., 1998; Sonoda et al., 2002]. In addition, well-conserved cysteine residues including Cys^{246}, which form intramolecular disulfide bonds, are in the N-terminal domains.

We performed *DSG4*, *LIPH*, and *LPAR6* gene mutation analysis and identified two prevalent missense mutations in the *LIPH* gene in the five independent Japanese ARH families. One mutation c.736T>A leads to an amino acid change within conserved cysteine residue that forms intramolecular disulfide bonds on the lid domain (p.Cys246Ser) (Fig. 2). The other mutation c.742C>A results in alteration of one amino acid of the catalytic triad (p.His248Asn) (Fig. 2B). These two residues, Cys^{246} and His^{248}, are highly conserved among *LIPH* of diverse species (Fig. 2A), suggesting that they play a critical role in enzyme activity. We speculate that these mutations drastically affect PA-PLA$_1\alpha$ activity.

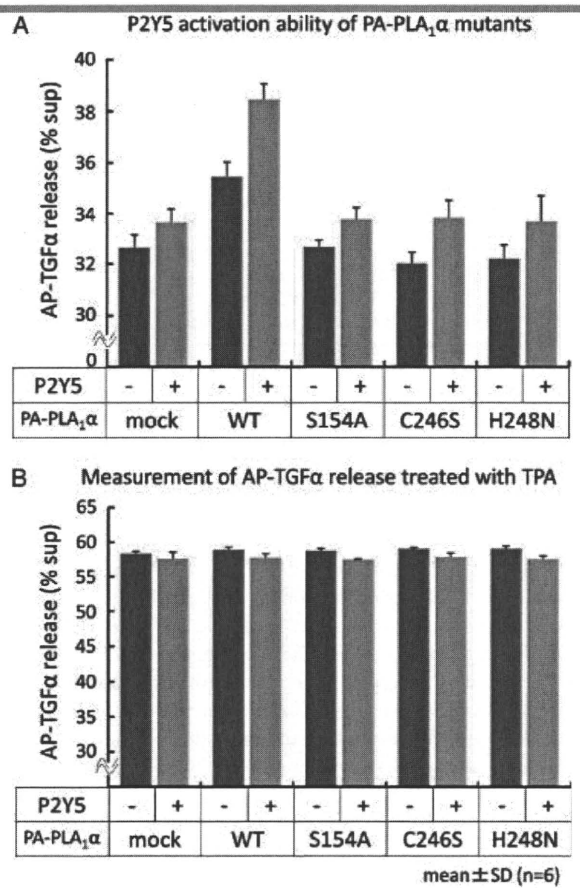


Figure 5. P2Y5 activation ability of PA-PLA$_1\alpha$ mutants. To monitor P2Y5 activation level by mutant and wild-type (WT) PA-PLA$_1\alpha$, we used *p*-nitrophenyl phosphate as a substrate for cleavage of AP-TGFα and measured the amount of AP-TGFα released from the HEK293 cells. **A:** The amount of free AP-TGFα produced by P2Y5 mock-transfected (P2Y5−) cells that were also transfected with WT PA-PLA$_1\alpha$ is significantly greater than that produced by P2Y5− cells transfected with an empty vector (mock). This indicates that HEK293 cells act to shed AP-TGFα, an activity that might be mediated by intrinsic LPA receptors. The amounts of AP-TGFα released from P2Y5-transfected (P2Y5+) cells expressing p.Ser154Ala (S154A), p.Cys246-Ser (C246S), or p.His248Asn (H248N) mutant PA-PLA$_1\alpha$ and P2Y5+ cells transfected with an empty vector (mock) are significantly lower than that from P2Y5+ cells expressing WT PA-PLA$_1\alpha$. **B:** TPA sheds AP-TGFα independently from the P2Y5 pathway. Effects of the TPA-induced shedding of AP-TGFα are similar in all the cells.

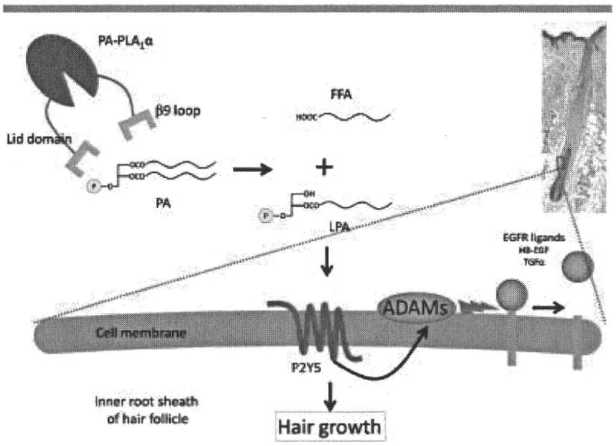


Figure 6. Schematic signaling pathways of LPA produced by PA-PLA$_1\alpha$ via the P2Y5 receptor. PA-PLA$_1\alpha$ hydrolyzes PA and produces LPA and FFA. LPA works as a ligand for P2Y5, a membrane-bound G-protein-coupled receptor. It has been documented that ADAM activation by P2Y5 results in ectodomain shedding of cell surface proteins including those of the EGF ligand family, such as HB-EGF and TGFα. These signal pathways are speculated to regulate proliferation and differentiation of inner root sheath cells of hair follicles. Abbreviations: PA, phosphatidic acid; FFA, free fatty acid; LPA, 2-acyl lysophosphatidic acid; ADAM, a disintegrin and metalloprotease; EGF, epidermal growth factor; HB-EGF, heparin binding EGF-like growth factor; TGFα, transforming growth factor-α.

So far, 14 *LIPH* gene mutations have been reported, four of which are prevalent [Ali et al., 2007; Horev et al., 2009; Jelani et al., 2008; Kamran-ul-Hassan Naqvi et al., 2009; Kazantseva et al., 2006; Nahum et al., 2009; Naz et al., 2009; Pasternack et al., 2009; Petukhova et al., 2009; Shimomura et al., 2009a,b,c]. One prevalent mutation, 985-bp deletion including exon 4 and the flanking introns, was detected in a large number of ARH patients from two ethnic groups, the Chuvash and Mari, in the Volga–Ural region of Russia [Kazantseva et al., 2006]. The ancestors of the Chuvash population settled in territory occupied by ancestral Mari populations. To determine the frequency of the mutant allele, they tested 2,292 chromosomes in the populations and found the *LIPH* deletion in populations of Chuvash (mutant allele frequency $P = 0.033$) and Mari (mutant allele frequency $P = 0.030$) origin. The mutant allele was restricted to these two populations and was not found in other Finno–Ugric populations or Russian populations from distant geographic regions [Kazantseva et al., 2006].

A deletion mutation exon7_8del has been identified in five consanguineous Pakistani families and 1 Guyanese family [Jelani et al., 2008; Petukhova et al., 2009; Shimomura et al., 2009b, 2009c]. A small deletion mutation 659_660delTA has been identified in several consanguineous Pakistani families and 1 Guyanese family [Jelani et al., 2008; Petukhova et al., 2009; Shimomura et al., 2009b,c]. Both mutations were defined as founder mutations shared in families from Pakistan and Guyana by haplotype analysis using microsatellite markers close to the *LIPH* gene [Jelani et al., 2008; Petukhova et al., 2009; Shimomura et al., 2009b,c]. In fact, these Guyanese families with ARH were descended from people who had come from India about 100 years ago, and it is plausible that both mutations originated from the Indian population [Shimomura et al., 2009c]. However, neither exon7_8del nor 659_660delTA mutations were detected in healthy control individuals of Pakistani origin and their minor allele frequencies were thought to be low in the Pakistani population [Jelani et al., 2008; Shimomura et al., 2009b].

All six of the Japanese ARH patients from the five families in the present study were compound heterozygous for c.736T>A (p.Cys246Ser) and c.742C>A (p.His248Asn) or homozygous for c.736T>A (p.Cys246Ser). c.736T>A (p.Cys246Ser) was found in all five families, and c.742C>A (p.His248Asn) was detected in four of the five families. Most recently, these missense mutations were identified in three Japanese ARH families [Shimomura et al., 2009a]. One family carries two heterozygous missense mutations, c.736T>A and c.742C>A, and the other two families are homozygous for the mutation c.736T>A. Thus, the missense mutations c.736T>A (p.Cys246Ser) and c.742C>A (p.His248Asn) are both suggested to be highly prevalent *LIPH* mutations in the Japanese population. In the previous article, however, screening assays with restriction enzymes excluded the existence of both

mutations in 100 unrelated healthy control individuals (200 alleles) of Japanese origin [Shimomura et al., 2009a]. In this study, in contrast, we used direct sequences and MASA analysis and identified these mutations in four alleles out of 200 unrelated control alleles (100 individuals) (minor allele frequency of c.736T>A, 3/200 $P = 0.015$; c. 742C>A, 1/200 $P = 0.005$; combined genotype, 4/200 $P = 0.020$). In addition, the present haplotype analysis revealed that the mutant alleles with c.736T>A and those with c.742C>A had specific haplotypes, respectively, which suggests that they derive from their own independent founders (Fig. 3, Table 1). From these results, we consider that the *LIPH* mutations c.736T>A (p.Cys246Ser) and c.742C>A (p.His248Asn) are extremely prevalent founder mutations for ARH in the Japanese population.

Previously, several deletion mutations and four missense mutations were reported in the *LIPH* gene [Ali et al., 2007; Horev et al., 2009; Jelani et al., 2008; Kamran-ul-Hassan Naqvi et al., 2009; Kazantseva et al., 2006; Nahum et al., 2009; Naz et al., 2009; Pasternack et al., 2009; Petukhova et al., 2009; Shimomura et al., 2009a,b,c]. In previous cases, ARH patients exhibited wide variability in the hypotrichosis phenotype, although most patients showed wooly hair during early childhood [Shimomura et al., 2009b]. Even ARH patients with identical *LIPH* gene mutations showed a wide variation in phenotype [Shimomura et al., 2009b]. In our cases, all the affected individuals had sparse, curled hair that grew slowly from birth and then stopped growing after reaching a few inches. There are no significant differences in clinical features between families and patients. We cannot exclude the possibility that differences in phenotype will emerge in the future, because our patients were still less than 10 years of age. The clinical features of the five families presented here are similar to those of families with the other mutations in the *LIPH* gene, and no apparent genotype/phenotype correlation was observed between the patients with deletion mutations and those with missense mutations.

PA-PLA$_1\alpha$ hydrolyzes PA and produces LPA and FFA concurrently [Sonoda et al., 2002]. The LPA that is produced by PA-PLA$_1\alpha$ acts as a ligand for P2Y5, one of the G-protein-coupled receptors (GPCRs), which has been identified as another causative gene for human hair growth deficiency [Pasternack et al., 2008; Shimomura et al., 2008]. It has been documented that ADAM activation by GPCRs introduces the ectodomain shedding of cell surface proteins, including the epidermal growth factor (EGF) ligand family whose members include heparin-binding EGF-like growth factor (HB-EGF) and TGFα [Ohtsu et al., 2006] (Fig. 6).

In this study, we performed two different in vitro PA-PLA$_1\alpha$ enzyme activity analyses. One involved analyzing PA-PLA$_1\alpha$ hydrolytic activity by measuring FFA (unpublished data). The p.Cys246Ser and p.His248Asn mutants showed complete abolition of PA-PLA$_1\alpha$ hydrolytic activity, comparable with supernatant of cells transfected with the empty vector only or with the control loss-of-function mutant carrying p.Ser154Ala. The other involved analyzing the P2Y5 activation ability of LPA produced by PA-PLA$_1\alpha$ by assaying free AP-TGFα (unpublished data). In this analysis, the p.Cys246Ser and p.His248Asn mutant PA-PLA$_1\alpha$ had no ability to activate P2Y5. These results clearly indicated that a loss of PA-PLA$_1\alpha$ function leads to defective activation of P2Y5 by LPA, resulting in ARH phenotype in ARH patients with *LIPH* mutations. Thus, complete loss of P2Y5 activation due to reduced LPA is thought to be involved in the pathogenesis of ARH.

While we were preparing the manuscript, Pasternack et al. [2009] reported that PA-PLA$_1\alpha$ derived from mutants with c.403_409 duplication frameshift mutation and in-frame mutations including c.280_369dup and c.527_628del did not show the enzymatic activity of converting PA to LPA in vitro, and that they did not activate P2Y5. The results presented in this study completely agree with their results, although the assay system for enzymatic evaluation and P2Y5 activation used by Pasternack et al. [2009] is quite different from ours. In addition, the affected amino acids in the mutant PA-PLA$_1\alpha$ analyzed in this study were quite different. Interestingly, our in vitro enzyme activity analysis revealed that the present two missense mutations strikingly affected the PA-PLA$_1\alpha$ activity as much as frameshift mutations and large deletion mutations like c.403_409 dup, c.280_369dup, and c.527_628del. These results were consistent with the fact that there is no significant difference in severity of hair loss between the present patients with missense mutations and affected individuals with frameshift mutations or large deletion mutations, c.403_409 dup, c.280_369dup, and c.527_628del. These results clearly indicated that the loss of PA-PLA$_1\alpha$ function caused by the two present mutations leads to defective activation of P2Y5 by LPA and suggest that loss of P2Y5 activation due to reduced LPA is involved in the pathogenesis of ARH.

Acknowledgments

We thank the patients for their generous cooperation and Ms. Akari Nagasaki, Ai Hayakawa, Yuko Hayakawa, and Shizuka Miyakoshi for their technical assistance on this project. This work was supported in part by Grant-in-Aid from the Ministry of Education, Science, Sports, and Culture of Japan to M. Akiyama (Kiban B 20390304) and by a grant from Ministry of Health, Labor and Welfare of Japan (Health and Labor Sciences Research Grants; Research on Intractable Diseases; H21-047) to M. Akiyama.

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Acta Derm Venereol 2010; 90: 621–624

CLINICAL REPORT

Localized Linear IgA/IgG Bullous Dermatosis

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Linear IgA/IgG bullous dermatosis (LAGBD) is an autoimmune blistering disease characterized by the local accumulation of IgA- and IgG-class anti-basement membrane autoantibodies. It typically presents as a generalized pruritic vesiculobullous eruption. No cases of localized LAGBD have yet been reported. We report a case of a 78-year-old man with LAGBD localized to the perianal area. The patient complained of suffering from persistent ulcers around the anus for more than 3 years. Physical examination revealed several blisters and ulcers up to 2-cm in diameter around the anus. No lesions were found elsewhere on the body. Histological analysis of a skin biopsy revealed subepidermal blistering, while direct immunofluorescence showed the linear deposition of IgA and IgG antibodies at the dermoepidermal junction. Indirect immunofluorescence of normal human skin whose layers had been separated using 1M NaCl showed the binding of both IgA and IgG to the epidermal side. Immunoblotting demonstrated the presence of circulating IgA and IgG autoantibodies that bound to a 120-kDa protein. This is the first case of localized LAGBD whose skin lesions were restricted to the perianal region. ***Key words: Linear IgA/IgG bullous dermatosis; linear IgA bullous dermatosis; bullous disease; BP180; collagen XVII; immunoblotting.***

(Accepted July 20, 2010.)

Acta Derm Venereol 2010; 90: 621–624.

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Linear IgA bullous dermatosis (LABD) is an autoimmune blistering disease characterized by subepidermal blisters and linear deposition of IgA autoantibodies at the dermoepidermal junction (1). In LABD, IgA autoantibodies most commonly recognize a 120-kDa or 97-kDa soluble ectodomain of collagen XVII or BP180 (2). Recently, linear IgA/IgG bullous dermatosis (LAGBD) was designated a subepidermal blistering disease in which linear deposition of both IgA and IgG anti-basement membrane autoantibodies occurs. It comprises a heterogeneous group of diseases, with the majority of cases being treated as a subgroup of LABD (3). Clinical features of both LAGBD and LABD vary. They usually present as annular vesiculobullous lesions over the whole body. Several cases of localized LABD have been described (4–7). In contrast, no cases of localized linear IgA/IgG bullous dermatosis (LAGBD) have yet been reported.

We herein describe a case of localized LAGBD in which the location of the lesions had been restricted for more than 3 years.

CASE REPORT

A 78-year-old man visited us complaining of a 3-year history of a perianal skin lesions. Topical steroid cream had not improved his condition. His medical history included stomach cancer (stage 1) and he had been treated for almost 10 years with a range of medicines, included teprenone, ursodeoxycholic acid and trimebutine maleate.

Physical examination revealed several ulcers and blisters on a well-demarcated, 5-cm plaque around the anus (Fig. 1). No skin lesions had appeared elsewhere on his body during the previous more than 3 years and the oral and ocular mucosae were not affected. Pruritus was absent, but defecation was painful. The rectal mucosa was unaffected and anal function was normal. A full blood count and tests of liver and renal function revealed no significant abnormalities, although the patient's HbA1c level was slightly raised (6.0%; normal range 4.3–5.8%).

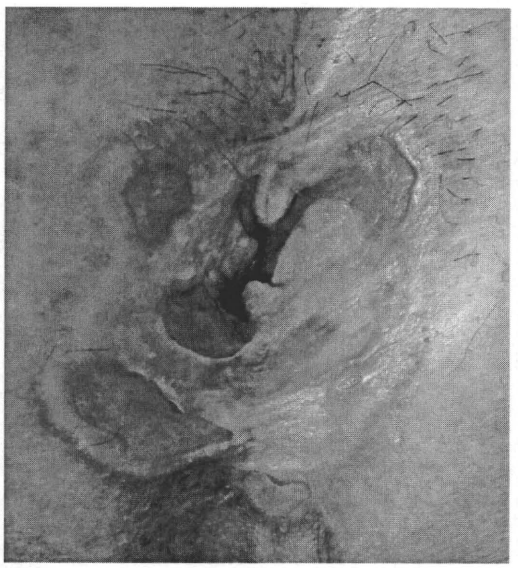

Fig. 1. Clinical manifestation. Ulcers on a well-demarcated plaque around the anus.

A biopsy was taken from the edge of the skin lesion. Histopathological analysis revealed subepidermal blistering and infiltration by lymphocytes, as well as neutrophils and eosinophils (Fig. 2a). Direct immunofluorescence (IF) analysis of lesional skin showed marked deposition of IgA (Fig. 2b), IgG (Fig. 2c) and C_3 at the dermo-epidermal junction. Indirect IF using 1M NaCl split human skin demonstrated whose layers had been separated with the binding of both IgA and IgG class autoantibodies to the epidermal side of the dermoepidermal junction. No dermal binding was observed. Epidermal extracts of normal human skin, supernatants of cultured HaCaT cells and recombinant proteins (NC16A and the C-terminal domain of BP180) were prepared as described previously (8–11) and used in immunoblot analysis of IgA and IgG class antibodies. While the patient's serum failed to react with 180- and 230-kDa antigens in epidermal extracts of normal human skin (Fig. 3a), it carried IgA and IgG class autoantibodies that bound to a 120-kDa protein in concentrated supernatants of cultured HaCaT cells (Fig. 3b). Immunoblotting with the recombinant NC16A domain of BP180 produced no specific binding (Fig. 3c). Further immunoblotting revealed that the patient's serum contained IgG, but not IgA, antibodies specific for the recombinant C-terminal domain of BP180 (BP915) (Fig. 3d). Based on these observations, we diagnosed the patient with LAGBD. Within 2 weeks of starting oral prednisolone treatment (0.5 mg/kg per day), the formation of new lesions ceased. When, however, the prednisolone dose was tapered to 0.1 mg/kg daily, new lesions again began to form. We therefore administered dapsone and gradually tapered the prednisolone dose. No new lesions appeared during 6 months of treatment with dapsone (25 mg daily).

DISCUSSION

LAGBD and LABD typically present as a generalized pruritic vesiculobullous eruption. Although 8 cases of localized LABD have been reported (4–7, 12–15), no cases of localized LAGBD have been described. We believe this to be the first case of localized LAGBD whose skin lesions were restricted to the perianal area.

LAGBD comprises an heterogeneous group of diseases characterized by subepidermal blistering and the specific binding of IgG and IgA antibodies to the epidermal basement membrane (3). Most patients carry autoantibodies against 97-/120-kDa antigens. Some, however, carry autoantibodies against a 230-kDa antigen, as well as additional, as yet uncharacterized epidermal antigens (3, 16). Some cases of LAGBD have been reported as LABD, even though they satisfy the criteria for diagnosis of LAGBD (17). Recent immunoserological studies detected circulating IgA and IgG autoantibodies specific for certain epitopes of BP180 in bullous pemphigoid and LABD patients (18, 19). These findings suggest that there may be considerable overlap between bullous pemphigoid, LABD and LAGBD. In only a few cases of LAGBD have clinical characteristics been described in detail. Most of these displayed a vesiculobullous appearance similar to that of LABD, and were effectively controlled with low-dose prednisolone, dapsone or sulfapyridine (16). More cases are needed to clarify the clinical differences between these disease categories.

The present case carried circulating IgA and IgG antibodies that bound to the 120-kDa soluble ectodomain of BP180. IgG class antibodies also reacted with BP915, the recombinant C-terminal domain of BP180,

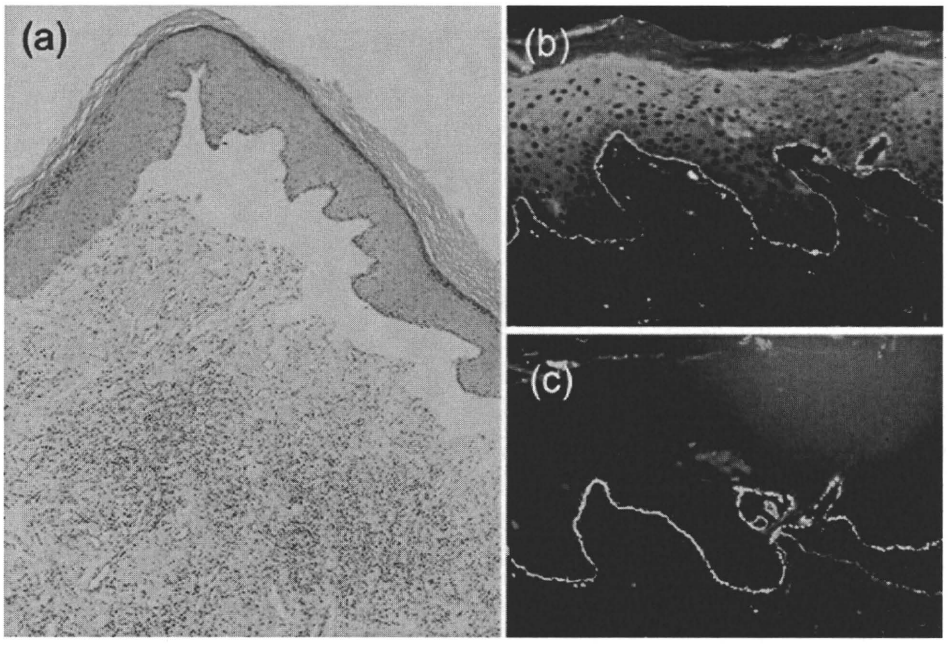

Fig. 2. (a) Biopsy of lesional skin revealed subepidermal blistering and lymphocytic infiltration. (Haemotoxylin&Eosin, original magnification ×100). (b, c) Direct immunofluorescence analysis of a lesional skin biopsy. Linear deposition of IgA (b) and (c) IgG at the dermoepidermal junction.

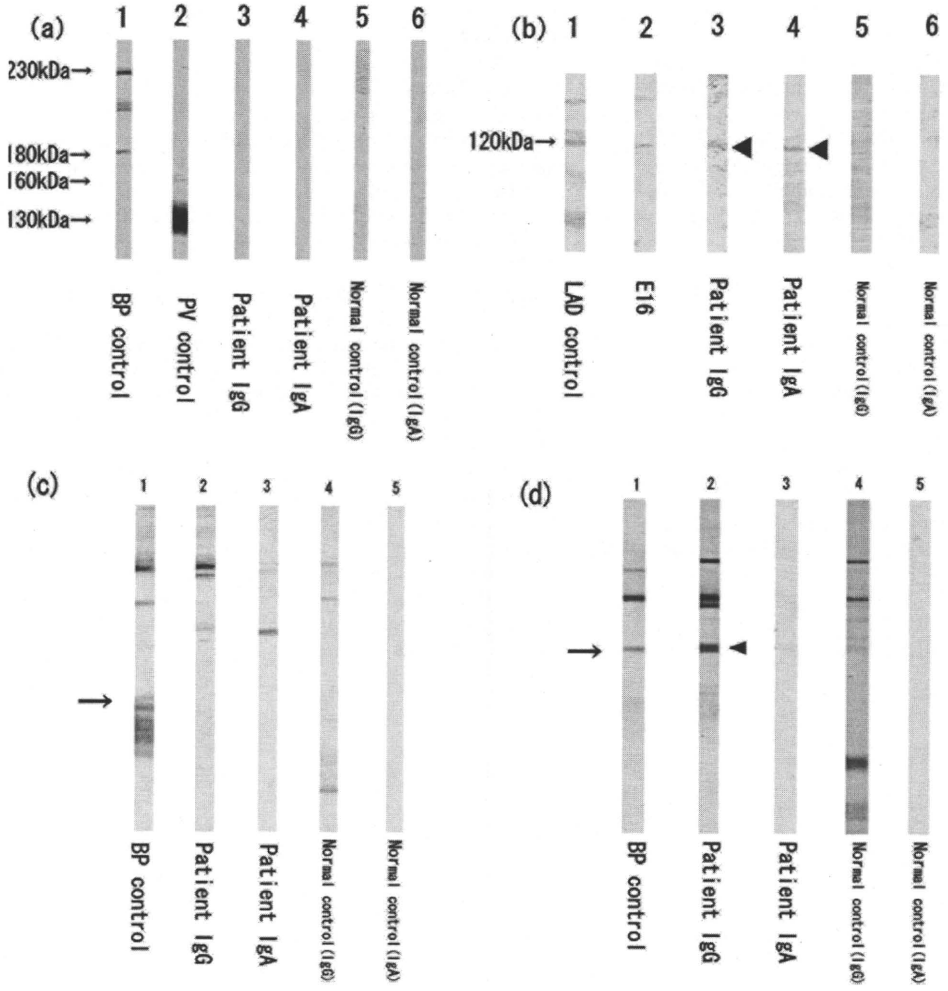

Fig. 3. (a) Immunoblotting using normal human epidermal extracts. Control bullous pemphigoid (BP) serum (lane 1) reacted with 180- and 230-kDa antigens. Control pemphigus vulgaris (PV) serum (lane 2) reacted with 130- and 160-kDa antigens. The patient's serum (lanes 3 and 4) and normal controls (lanes 5 and 6) showed no reactivity. (b) Immunoblotting using conditioned medium of cultured HaCaT cells. Control linear IgA bullous dermatosis (LABD) serum (lane 1), E16 (a goat polyclonal antibody raised against the C-terminus of BP180; Santa Cruz) (lane 2), and the patient's IgG and IgA antibodies (lanes 3 and 4, arrowheads) reacted with a 120-kDa antigen. Normal control samples (lanes 5 and 6) showed no reactivity with the 120-kDa antigen. (c) Reactivity with the NC16A domain of BP180 (arrow) (lane 1). Patient IgA and IgG (lanes 2 and 3) and normal control (lanes 4 and 5) showed no reactivity in immunoblotting analyses. (d) Reactivity with BP915 (recombinant C-terminal domain of BP180). A bullous pemphigoid control (arrow) (lane 1) and the patient's IgG (arrowhead) (lane 2) showed reactivity (lane 1, 2) in immunoblotting analyses. The patient's IgA (lane 3) and normal controls (lanes 4 and 5) showed no reactivity.

which is the protein that is primarily targeted in mucous membrane pemphigoid (MMP) (11). In a study by Georgi et al. (17) BP915 was bound by IgA in 44% of lamina lucida-type LABD serum samples tested and by IgG in 33% of such samples (17). The authors concluded that the C-terminus of BP180 represents the primary target on the BP180 ectodomain for both IgA and IgG antibodies in the serum of LABD patients (17). Healing with scarring is characteristic of MMP but it is also found in some lamina lucida-type LABD patients (17, 20). This clinical overlap may well be explained by overlap in the antigenic sites on the BP180 ectodomain targeted by autoantibodies.

In summary, we have described an unusual case of LAGBD localized to the perianal region for more than 3 years that reveals the heterogeneous nature of LAGBD. LAGBD should be considered when diagnosing the cause of perianal blistering.

ACKNOWLEDGEMENT

We thank Ms Yuko Hayakawa for her technical assistance.

The authors declare no conflict of interest.

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Medical genetics

An Indian family with Sjögren-Larsson syndrome caused by a novel *ALDH3A2* mutation

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Abstract

Sjögren-Larsson syndrome is an autosomal-recessive hereditary disorder characterized by congenital ichthyosis, mental retardation and spastic diplegia or tetraplegia. It is known that mutations in the fatty aldehyde dehydrogenase (FALDH) gene (*ALDH3A2*) underlie SLS. We report two Indian sisters showing typical clinical features of SLS. Direct sequencing of the entire coding region of *ALDH3A2* revealed a novel homozygous mutation, c.142G>T (p.Asp48Tyr) in exon 1, in both patients. Their parents harbored the mutation heterozygously. Mutant-allele-specific amplification analysis using PCR products as a template verified the mutation in the patients. The aspartic acid residue at the mutation site is located in the C-terminal portion of the second a-helix strand, a2, of N-terminal four helices of FALDH and the FALDH amino-acid sequence alignment shows that this aspartic acid residue is conserved among several diverse species. Until now, a number of mutations in *ALDH3A2* have been shown to be responsible for SLS in Europe, the Middle East, Africa, and North and South America. However, in Asian populations, *ALDH3A2* mutations have been identified only in Japanese SLS patients. Here we report an *ALDH3A2* mutation for the first time in SLS patients in the Asian country other than Japan. The present results suggest that *ALDH3A2* is a gene responsible for SLS in Asian populations. We hope *ALDH3A2* mutation search will be globally available including many Asian countries in the future.

Case

Two sisters were born in an Indian nonconsanguineous family. The patient was a 1.5-year-old girl. She had had severe ichthyosis on the entire body since birth, especially prominent on the bilateral lower limbs (Fig. 1a–c). She showed mental retardation and spastic tetraplegia. Ocular fundus evaluation revealed white dots in the maculae. The elder sister also had icthyotic lesions all over the body at birth and had global developmental delay. She had had seizures since 2.5 years of age that had been controlled with multiple antiepileptic medications. At the age of four, severe hyperkeratosis appeared on the chest, back, axillae and predominantly over the limbs (Fig. 1d,e). She has hypertelorism, dolichocephalic head, large low-set ears, long eyelashes and short 3rd, 4th, and 5th metatarsals. Neurological evaluations revealed severe spastic tetraplegia with persistent ankle clonus and complete head lag. She showed serious mental retardation. She had severe photophobia, and ocular fundus evaluation showed white glistening dots in the maculae bilaterally. Severe auditory startle reaction was a characteristic feature. Magnetic resonance imaging of the brain showed bilateral symmetrical diffuse white matter at high intensity in T2-weighted images in the frontal, temporal, and parietal regions. Both sisters were diagnosed with Sjögren-Larsson syndrome (SLS) from these clinical features and laboratory data.

Fatty aldehyde dehydrogenase (FALDH) gene (*ALDH3A2*) mutational analysis was performed on the affected girls and their parents, as previously described.[1,2] In the patients, a novel homozygous mutation, c.142G>T (p.Asp48Tyr) in exon 1, was identified. Their parents harbored the mutation heterozygously (Fig. 2a). This mutation was not found in 200 normal unrelated alleles (100 individuals) by direct sequence analysis. Mutant-allele-specific amplification (MASA) analysis verified the mutation in this family (Fig. 2b).

Discussion

Sjögren-Larsson syndrome (MIM# 270200) is an autosomal-recessive hereditary disorder characterized by congenital ichthyosis, mental retardation and spastic diplegia or

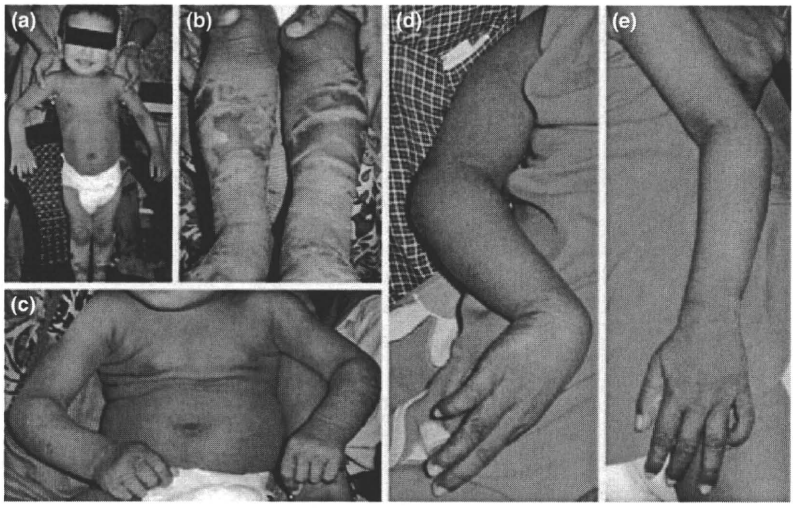

Figure 1 Clinical features of the Indian sisters with SLS. (a–c) The younger sister. Hyperkeratosis and scales cover whole body surface at 1.5 years of age (a). Dark brown scales are seen on the bilateral legs (b), the arms and the trunk (c). (d, e) The elder sister shows hyperkeratosis and brown scales on the bilateral arms at 4 years of age

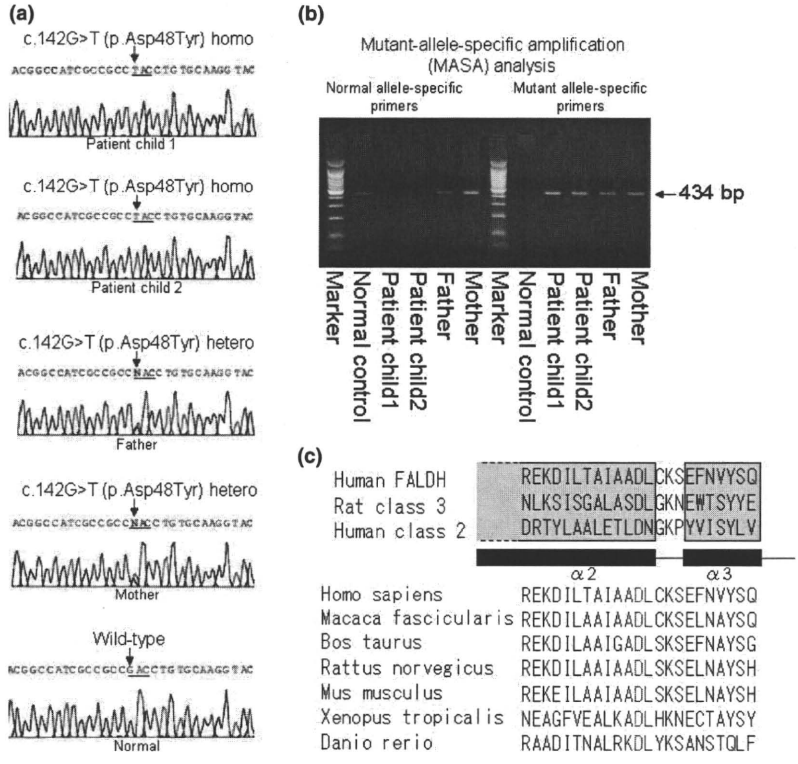

Figure 2 *ALDH3A2* mutation in the present SLS patients, and sequence alignments around the missense mutation. (a) Sequence analysis of *ALDH3A2*. In both patients, the younger sister (child 1) and the elder sister (child 2), a homozygous missense mutation c.142G>T (p.Asp48Tyr) in exon 1 derived from their parents was detected. The parents were heterozygous for the mutation. (b) Mutant allele-specific amplification analysis. With normal allele-specific primers, no amplification band is seen in the PCR products from the patients' DNA samples, suggesting that they have no normal allele. With mutant allele specific primers, the amplification band from the mutant alleles is detected as a 434-bp fragment in the PCR products from the DNA samples from the patients and their parents, and not in the PCR products from control DNA samples. This confirms the presence of the mutation c.142G>T in the patients. (c) Top: a sequence alignment between FALDH, rat class 3 and human class 2 ALDHs. Aspartic acid residue at codon 48 of FALDH is conserved. Secondary structure components found in the class 3 rat ALDH structure by Liu *et al.*[6] are presented with bars representing α-helices. Bottom: FALDH amino acid sequence alignment shows the level of conservation in diverse species of aspartic acid residue at codon 48 (D48) (red characters), which was altered by the missense mutation in the present family

tetraplegia.[3] In 1996, De Laurenzi *et al.*[4] reported that mutations in *ALDH3A2* underlie SLS. The present study reports a novel homozygous mutation in *ALDH3A2* in an Indian family with SLS.

The FALDH amino-acid sequence alignment shows that this aspartic acid residue at codon 48 is conserved among several diverse species. Compared with other aldehyde dehydrogenase (ALDH)-related sequences identified by Perozich *et al.*,[5] this aspartic acid is highly conserved among many members of the ALDH family (Fig. 2c). Analysis of the crystallized 3-D structure of the related class 3 rat cytosolic ALDH revealed that this aspartic acid is located in the C-terminal portion of the second α-helix strand, α2, of N-terminal four helices (Fig. 2c).[6] These findings strongly suggest that this aspartic acid residue is essential for the normal function of the FALDH. In the literature, missense mutation p.Ile45Phe in the α2 helix, three codons upstream of the present mutation site, was reported and the mutant enzyme was revealed to have only 9% residual enzyme activity compared with the wild-type enzyme.[7]

Until now, a number of mutations in *ALDH3A2* have been shown to be responsible for SLS in Europe, the Middle East, Africa, and North and South America.[1,7] However, in Asian populations, *ALDH3A2* mutations have been identified only in Japanese SLS patients.[1,2,8–10] Here, we report an *ALDH3A2* mutation for the first time in SLS patients in the Asian country other than Japan. The present results suggest that *ALDH3A2* is a gene responsible for SLS in Asian populations. Mutation analysis of the *ALDH3A2* gene is a highly sensitive method of confirming a diagnosis of SLS. It does not require a skin biopsy or FALDH enzymatic assays. We hope *ALDH3A2* mutation search will be globally available including many Asian countries in the future.

Acknowledgments

We thank Ms Akari Nagasaki, Ms Megumi Sato and Ms Yuki Miyamura for their technical assistance. This work was supported in part by a Grant-in-Aid from the Ministry of Education, Science, Sports, and Culture of Japan to M. Akiyama (Kiban B 20390304) and by a grant from Ministry of Health, Labour and Welfare of Japan (Health and Labour Sciences Research Grants; Research on Intrac\Diseases; H21-047) to M. Akiyama.

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Generalized exacerbation of systemic allergic dermatitis due to zinc patch test and dental treatments

Contact Dermatitis 2010: 62: 372–373

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Key words: Dental metal fillings; lymphocyte stimulation test; patch test; systemic allergic dermatitis; zinc.

Nickel, gold, palladium, and chromate are well-known allergens; however, zinc is much less commonly reported as an allergen (1).

We report a severe case of systemic allergic dermatitis to zinc in which generalized flare-up reactions occurred from a zinc patch test, as well as with each replacement of the patient's zinc-containing dental fillings.

Case Report

A 37-year-old Japanese man was referred with a one-year history of multiple pruritic eruptions covering his entire body. The skin lesions had been refractory to topical corticosteroids and anti-histamines. Examination showed an oedematous erythema with lesions 3–10 mm in diameter that were distributed over his entire body. A detailed history revealed that the patient had had dental fillings 3 months prior to the onset of the rash.

We suspected systemic allergic dermatitis because of the dental fillings. We performed patch test with a metal series (Torii Pharmaceutical Co., Ltd., Tokyo, Japan) consisting of aluminium chloride, gold chloride, tin chloride, iron chloride, platinum chloride, palladium chloride, indium chloride, manganese chloride, silver bromide, cobalt chloride, potassium dichromate, nickel sulfate, and zinc chloride. He developed a positive (+) reaction (ICDRG criteria) to zinc chloride on D2 (Table 1) and this persisted to D7. During patch testing, the skin lesions dramatically flared at previous lesion sites (Fig. 1). Serum zinc concentration was within normal limits; there was an eosinophilia. Lymphocyte stimulation test revealed a reaction to zinc chloride with a stimulation index of 518% (normal is <180%). A skin biopsy from erythema lesion on the back showed spongiosis and perivascular lymphocytic infiltration. Based on these clinical and histological findings, we diagnosed his pruritic eruption as systemic allergic dermatitis because of zinc.

Dental inspection showed that he had 11 teeth with zinc-containing metal fillings. We have been removing the fillings one by one, but with each

Table 1. Results of patch testing to 13 dental allergens

	D2	D3	D7
Zinc chloride 2.0% pet.	+	+	+
Other 12 dental allergens	–	–	–

Conflict of interests: The authors have declared no conflicts.