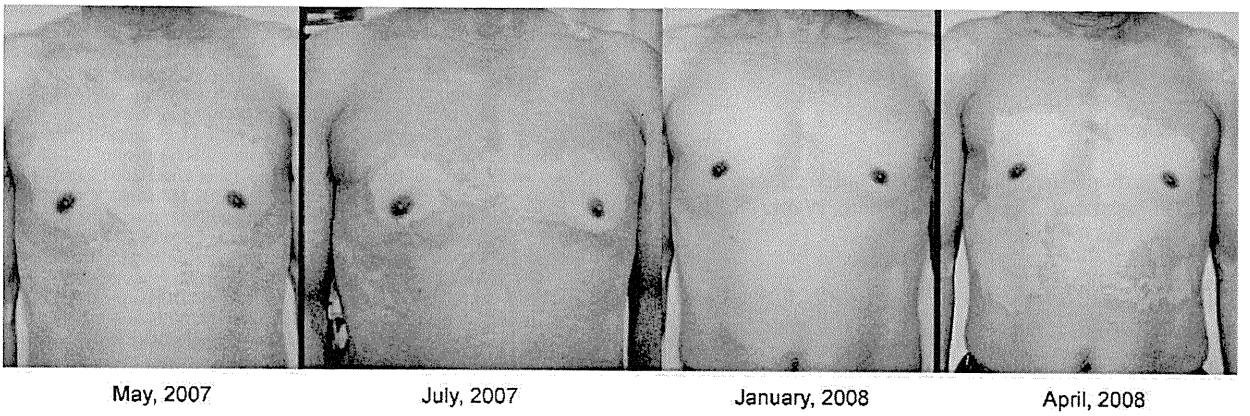


**Fig. 5.** Distinct seasonal variation of ichthyosis. Ichthyosiform lesions were aggravated from spring and formed erythroderma throughout hot season, but ameliorated in winter.

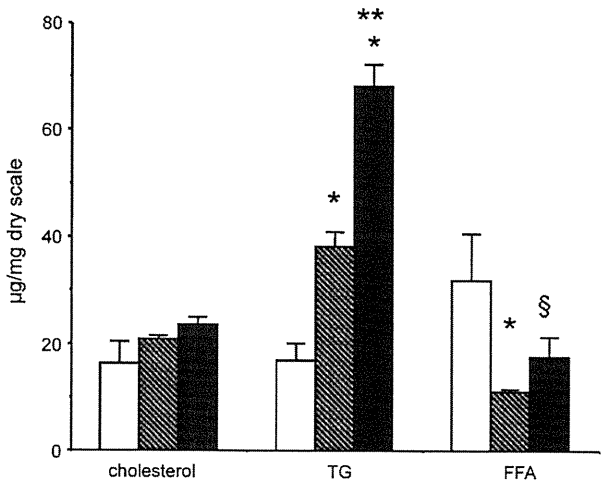


**Fig. 6.** Lipid analysis of scales taken from sunburn lesion as controls (white bar), patient's lesions at aggravation and remission stages (black, shaded bars, respectively), showing positive correlation of increased TG levels with disease severity. FFA levels are invariably decreased in patient's scales. Mean ± s.d. μg/mg dry scale. $^{*}p < 0.01$ vs. control. $^{**}p < 0.01$ vs. remission stage. $^{\S}p < 0.02$ vs. control.

contrast, free fatty acids were invariably decreased in the patient's scales compared with control. These results were relevant, since DCS is characterized by dysfunction of CGI-58, an activator of ATGL, which hydrolyzes TG to release FFA.

## 4. Discussion

Dorfman–Chanarin syndrome (DCS) is defined as a neutral lipid storage disorder with ichthyosis (NLSDI), which is attributed to mutations in CGI-58. Since CGI-58 is an activator of adipose triglyceride lipase (ATGL), patients with DCS demonstrated systemic storage of triglycerides as found in patients with mutations in ATGL, which were designated a neutral lipid storage disorder with myopathy (NLSDM). However, NLSDM patients and ATGL-deficient mice did not develop ichthyosis [17,18]. It was, therefore, hypothesized that CGI-58 could have an additional metabolic function required for normal skin physiology [17]. This hypothesis was supported by the fact that the lipid accumulation in the epidermis was higher in DCS than in NLSDM [17].

Sequencing analysis of the *CGI-58* gene using the patient's blood cells revealed a novel missense mutation in nucleotide position 215 (T>C) within the exon 3. As far as we examined, however, no mutation of this gene was found in another allele. Moreover, RT-PCR using primer sets specific for some exons including the exon 3 demonstrated no mRNA alteration in quantity and size compared to healthy controls (data not shown). Therefore, it was unlikely that truncation or instability of this gene occurred in our patient. His family history suggested that the inheritance was recessive, although there was no DCS patient among his relatives. It remained unsolved whether he had another undefined mutation of the *CGI-58* in another allele (compound heterozygous) or whether he harboured any posttransriptional alteration in this gene. Recent findings of the *CGI-58* gene expanded the clinical and mutational spectrum and underlie the genetic heterogeneity of this disease [21]. Alternatively, unknown mutations of other gene including the *ATGL* gene, might play a role [10], since mutations of *CGI-58* were not invariably found in clinically typical patients with DCS [15,17].

In the present study, a lipid analysis of scales from the DCS patient revealed elevated TG levels in the epidermis, which was correlated with the clinical severity of ichthyosis. A decrease in FFA levels in the epidermis could be also attributed to a dysfunction of ATGL in this patient. Since cholesterol, ceramides, and FFA comprise intercellular lipids of the stratum corneum, a decrease in FFA levels could affect the integrity of the cornified layer [22,23]. These abnormalities might contribute to development of ichthyosis in this patient. It is, however, unclear why permeability barrier remained intact in our patient, while the water holding capacity was markedly decreased in the involved lesions. Akiyama and coworkers recently demonstrated that CGI-58 knockdown reduced expression of keratinocyte differentiation markers including filaggrin [13], which plays an important role in retaining stratum corneum water.

New pieces of puzzle come from the finding of seasonal fluctuation of ichthyosis severity in this patient and a previous case [8], presumably depending on the environmental temperature. In both cases, summer was an aggravating season. Elevation of temperature might affect the ATGL activity, if any left, or other lipolysis pathways, for example, the hormone sensitive lipase-dependent pathway [18]. Therefore, it should be clarified how the mutated *CGI-58* in our patient impaired the ATGL, and whether ATGL, if any, or other compensatory lipase activity were temperature-sensitive. Haemmerle et al. have demonstrated that $ATGL^{-/-}$ mice showed defective cold adaptation because they could not produce FFAs to fuel thermogenesis [18]. We assume that ATGL activity could be decreased upon elevated environmental temperature because relatively reduced requirement of FFAs to be provided. In a patient like our case, impaired CGI-58 activity further might attenuate the ATGL activity at high temperature. To

verify this, lipase activity assay from the patient's cells will be required to see whether heat stimulation worsens it, which would be reversed by introduction of wild-type *CGI-58* gene.

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# Neutral Lipid Storage Leads to Acylceramide Deficiency, Likely Contributing to the Pathogenesis of Dorfman–Chanarin Syndrome

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## TO THE EDITOR

Dorfman–Chanarin syndrome (DCS) is an autosomal recessive, neutral lipid storage disorder with ichthyosis (NLSDI) due to loss-of-function mutations in *CGI-58* (a/β-hydrolase domain-containing protein 5, *ABHD5*). *CGI-58* encodes a 39 kDa protein, a widely expressed cofactor in mammalian tissues including epidermis that activates adipose triglyceride (TG) lipase (reviewed in Schweiger *et al.*, 2009; Yamaguchi and Osumi, 2009), as well as other still unidentified TG lipases (Radner *et al.*, 2009). CGI-58 expression increases during keratinocyte differentiation; and conversely, knockdown of this cofactor reduces keratinocyte differentiation (Akiyama *et al.*, 2008). Epidermal permeability barrier defects due in part to lamellar/nonlamellar phase separation of secreted lipids, within extracellular domains of the stratum corneum (SC) have been proposed to account for the barrier abnormalities in NLSDI (Elias and Williams, 1985; Demerjian *et al.*, 2006). Although defective extracellular lipid organization clearly is one contributor (Demerjian *et al.*, 2006), we assessed whether diversion of free fatty acid (FA) into esterified lipids causes lipid abnormalities that further impact barrier formation.

We recently reported a DCS patient with a, to our knowledge, previously unreported *CGI-58* missense mutation (Ujihara *et al.*, 2010), exhibiting abnormal barrier-related structures resembling other NLSDI patients (Demerjian *et al.*, 2006). Both mild and severely affected ichthyotic SCs revealed increased TG and decreased FA levels in comparison with SC fraction from normal subjects (Ujihara *et al.*, 2010). Pertinently, the extent of the increase in TG levels correlated with site-specific differences in the severity of the dermatosis (Ujihara *et al.*, 2010). These observations suggest that divergence of FA to TG contributes to disease phenotype in NLSDI.

We previously showed that ω-*O*-acylceramides (or acylCer) that have only been identified in differentiated layers of epidermis in terrestrial mammals are essential constituents of the epidermal permeability barrier; that is, lack of acylCer formation results in neonatal death due to abnormal epidermal permeability barrier function (Vasireddy *et al.*, 2007). AcylCer and the de-ω-*O*-esterified form (ω-hydroxy [ω-OH] ceramide [Cer]) are present either as free (unbound) or bound species (Uchida and Holleran, 2008). The latter form a continuous lipid monolayer, the corneocyte-bound lipid envelope (CLE); that is, a pool of ω-OH Cer, which is covalently bound to the external surface of the cornified envelope (Uchida and Holleran, 2008). Although free acylCer are critical for the formation of the lamellar membranes (Bouwstra *et al.*, 1998), our previous studies suggest the CLE is also important for normal permeability barrier function (Behne *et al.*, 2000). Prior studies suggest that FAs derived from TG are used in the ω-*O*-esterification step to form acylCer (Wertz and Downing, 1990). Moreover, TG, linoleate, and acylCer, but not phosphoglycerolipid content, decline in acyl-CoA: diacylglycerol acyltransferase-2-deficient mice, which also show a permeability barrier abnormality (Stone *et al.*, 2004). In addition, linoleate, which is the predominant FA that is used for ω-*O*-esterification, is enriched in TG in mouse skin (Stone *et al.*, 2004). Thus, we hypothesized that a failure of TG hydrolysis, due to abnormal CGI-58 function, could attenuate permeability barrier formation in NLSDI by decreasing acylCer content of affected SC.

Therefore, we first investigated the lipid profiles in solvent extracts of SC from this DCS patient (Ujihara *et al.*, 2010). Neither cholesterol (Ujihara *et al.*, 2010) nor total (bulk) Cer (Figure 1a) content was altered. Yet, Cer comprises a family of at least 10 species in humans (Uchida and Holleran, 2008). Because not only bulk Cer amount but also each individual Cer species contributes to the formation of competent lamellar structures required for barrier function (Bouwstra *et al.*, 1998), we subfractionated Cer into individual Cer species. Whereas the major Cer subfractions, NS (Cer 2), NP (Cer 3), and AS (Cer 5) were not significantly altered, acylCer were present at only trace levels in the patient sample (Figure 1b; because sample amounts were limited, other minor Cer species; that is, EOH (Cer 4), AP (Cer 6) (AP), AH (Cer 7) could not be quantitated) (abbreviations for Cer structures are according to Motta. *et al.* and Robson K. *et al.*, details reviewed in Uchida and Holleran, 2008).

*Abbreviations: acylCer, acylceramide; Cer, ceramide; CGI-58, Comparative Gene Identification-58; CLE, bound lipid envelope; DCS, Dorfman–Chanarin syndrome; FA, fatty acid; KC, keratinocyte; NLSDI, neutral lipid storage disorder with ichthyosis; ω-OH, omega hydroxy; SC, stratum corneum; TG, triacylglyceride*

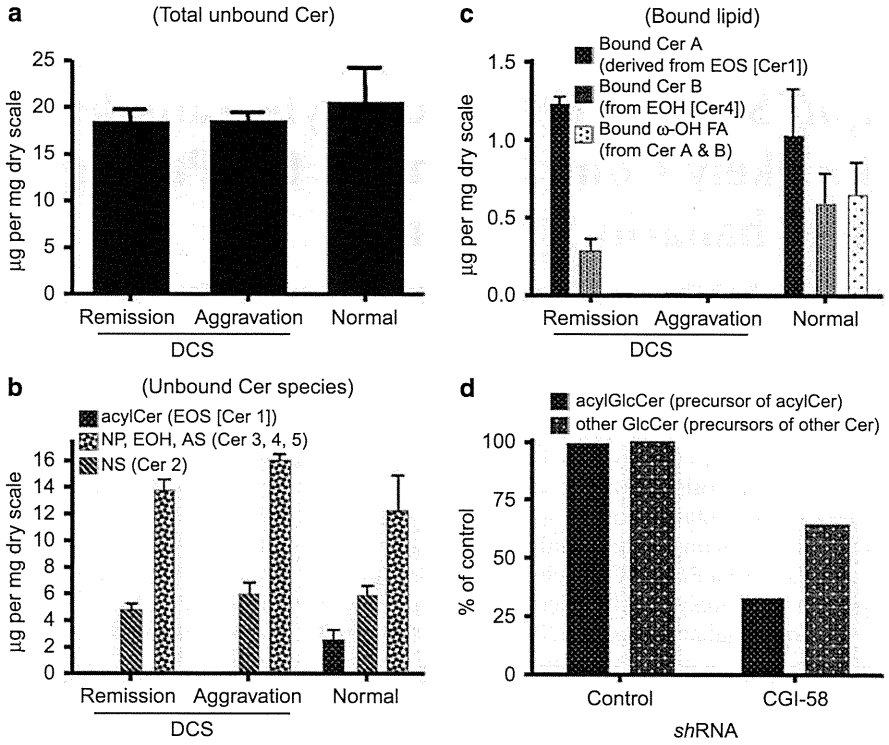


**Figure 1. Lipid profile in the stratum corneum.** Both unbound acylCer and bound ω-OH Cer deficiencies occur in a Dorfman–Chanarin syndrome (DCS) stratum corneum (SC) (**a–c**), whereas diminished CGI-58 expression decreases acylglucosylCer production (**d**). Normal SC from sunburn lesion as controls. CHK transfected with lentivirus-expressed shRNA (CGI-58 or control vector) were cultured in differentiation-inducing medium (Uchida *et al.*, 2001). Lipids were isolated from SC or cells and quantitated using thin-layer chromatography-scanning densitometry as described previously (Uchida *et al.*, 2001). $n=1$ (DC) and $n=3$ (normal). All studies were approved by the institutional ethics review boards (Kochi University and University of California, San Francisco) and were performed according to the Declaration of Helsinki Principles.

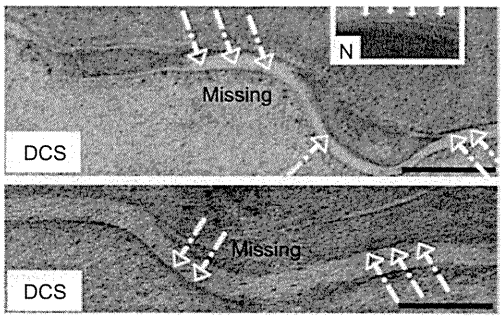


**Figure 2. Electron micrographs display lack of continuous lipid monolayer, CLE, in the SC from two DCS patients (Demerjian *et al.*, 2006) vs. normal subject (inset, N).** Skin samples were fixed in Karnovsky's fixative, and post-fixed with ruthenium tetroxide or osmium tetroxide, as previously described (Behne *et al.*, 2000). Ultrathin sections were examined, after further contrasted with lead citrate, with a Zeiss 10A (Carl Zeiss, Thornwood, NY) (Behne *et al.*, 2000). Arrows (with solid line, presence and with dotted line, absence) indicate CLE structures. Bars = 100 nm.

Not only bound ω-OH Cer, but also bound ω-OH FA (resulting from the subsequent hydrolysis of some bound ω-OH Cer by ceramidase) decline significantly in DCS (Figure 1c). As with TG accumulation (Ujihara *et al.*, 2010), the decrease in these bound lipids reflects disease severity. Accordingly, this patient, as well as in two additional DCS patients (Demerjian *et al.*, 2006), lacked CLE on ultrastructural analysis of affected SC (Figure 2).

Finally, we investigated whether the decreased acylCer in DCS is due to a gain-of-function of mutation in *CGI-58*, rather than a deficiency of TG-derived FA. A substantial decrease in acylglucosylCer (=acylCer precursor), but not in other glucosylCer species, was evident in lentivirus-expressed CGI-58 shRNA-treated cultured human keratinocytes (Figure 1d). Hence, by facilitating the lipolysis of TG, CGI-58 provides FA for ω-*O*-esterification leading to acylCer formation. The recent demonstration of a lethal, postnatal permeability barrier defect and deficiency of both acylCer and bound ω-OH Cer in *cgi-58*-null mice (Radner *et al.*, 2009) further supports this conclusion.

We conclude from these and previous studies that CGI-58 not only facilitates TG lipolysis but also provides FA for the ω-*O*-esterification of Cer leading to acylCer production, as well as bound ω-OH Cer generation leading to CLE formation (see Supplementary Figure S1 online). These studies highlight that the deficiency of an essential barrier constitute, acylCer, likely contributes to the permeability barrier

abnormality in DCS. Although the function of the CLE is still unclear, a role as a necessary scaffold for the lamellar bilayer organization is likely (Uchida and Holleran, 2008). Thus, CLE deficiency, coupled with disorganization of extracellular lamellar bilayers, likely merge to provoke the barrier abnormality in NLSDI (see Supplementary Figure S2 online). Finally, to overcome this metabolic disadvantage in forming the epidermal permeability barrier, epidermal proliferation likely increases, which in turn results in hyperkeratosis, phenotypic features common to virtually all of the ichthyoses (Demerjian *et al.*, 2006; Akiyama *et al.*, 2008), that is, 'A compromised permeability barrier 'drives' the hyperproliferative epidermis in NLSDI and other ichthyoses' (Elias *et al.*, 2008).

**CONFLICT OF INTEREST**
The authors state no conflict of interest.

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**SUPPLEMENTARY MATERIAL**

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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# Detection of Human Papillomavirus DNA in Plucked Eyebrow Hair from HIV-Infected Patients

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## TO THE EDITOR

The risk of developing human papillomavirus (HPV)-related benign and malignant cutaneous lesions is markedly increased in immunosuppressed people such as organ-transplant recipients (Harwood *et al.*, 2000) and HIV-infected patients (Grulich *et al.*, 2007; Stier and Baranoski, 2008). Although HPV DNA in plucked eyebrow hair has been well investigated (Boxman *et al.*, 1997) in renal transplant recipients and immunocompetent patients (ICPs) and correlated with both benign and malignant cutaneous lesions (Struijk *et al.*, 2003; Plasmeijer *et al.*, 2009), very little is known about HPV prevalence in eyebrow hair from HIV patients.

The study design was approved by the research ethics committee and all

*Abbreviations: HPV, human papillomavirus; ICP, immunocompetent patient*

BJD
British Journal of Dermatology

# Three-base deletion mutation c.120_122delGTT in *ATP2A2* leads to the unique phenotype of comedonal Darier disease

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**Conflicts of interest**
*None declared.*

*The first two authors contributed equally to this work.*

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Darier disease (DD; Darier–White disease; OMIM 124200) is an autosomal dominant inherited disorder.[1] Clinically, it is characterized by recurrent and multiple hyperkeratotic papules or nodules affecting the trunk and flexural aspects of the extremities.[1] Characteristic histopathological features are dyskeratotic cells in the form of corps ronds and grains, suprabasal acantholysis forming suprabasal lacunae and irregular upward proliferation into the lacunae of papillae lined with a single layer of basal cells, the so-called villi.[2] The causative gene is *ATP2A2* (OMIM 108740) on chromosome 12, which encodes the sarco/endoplasmic reticulum calcium pump ATPase (SERCA2).[2]

Clinical variants include the hypertrophic, vesiculobullous, hypopigmented, cornifying, zosteriform and linear subtypes, and the rare subtype comedonal Darier disease (CDD).[1,3–6] CDD tends to appear in seborrhoeic areas. The characteristic morphological features are prominent follicular involvement, sometimes associated with keratotic plugs, and the presence of greatly elongated dermal villi and papillary projections.[4] There have been no conclusive reports on the aetiology of CDD and it is still controversial as to whether or not CDD is a variant of DD, and if it is caused by *ATP2A2* gene mutations, although a combination of CDD and classic DD was reported in one patient.[7] The present study identifies a previously unreported three-base deletion mutation in *ATP2A2* in a patient with CDD.

## Patient and methods

A 22-year-old Japanese man presented with acne-like comedonal lesions on the face and chest, most densely distributed on the forehead, cheeks, back, axillae and chest. The comedonal lesions had first appeared in his teens and had gradually increased in number. Physical examination showed open comedones, closed comedones, red papules, nodules, cysts and ice-pick scars (Fig. 1a,b). His parents were clinically healthy, without any skin problems. He had been treated with oral biotin, Korean ginseng, an antihistamine, topical bufexamac ointment, calcipotriol ointment and betamethasone butyrate propionate ointment without any improvement. Histopathological observations revealed suprabasal acantholytic clefts and numerous dyskeratotic cells (corps ronds) in the outer root sheath in the affected follicular infundibulum, which was surrounded by plasma cells and lymphocytes (Fig. 1c,d). We made a diagnosis of CDD. Oral etretinate 10 mg daily combined with adapalene gel remarkably improved most of the skin lesions, except those on the forehead.

Polymerase chain reaction (PCR) amplification and direct sequencing of the entire coding region and exon/intron boundaries of *ATP2A2* were performed using the proband's and his parents' genomic DNA samples and genomic DNA samples from 50 healthy Japanese individuals as controls. A detected mutation was verified by mutant allele-specific amplification analysis[8] with mutant allele-specific primers carrying the substitution of two bases at the 3′ end, a PCR product band derived from the mutant allele.

This study was approved by the Hokkaido University Medical Ethics Committee and conducted according to the principles of the Declaration of Helsinki. All clinical samples were obtained with informed consent.

## Results and discussion

Direct sequencing of *ATP2A2* in the proband's genomic DNA revealed a heterozygous three-base deletion c.120_122delGTT in exon 2, which causes deletion of leucine at the 41st amino acid residue from the amino terminus (p.Leu41del). This mutation was not detected in his parents nor in 100 normal unrelated alleles from 50 healthy individuals (Fig. 2). No other pathogenic mutations were detected within *ATP2A2* in the patient's DNA. By mutant allele-specific amplification ana-

 

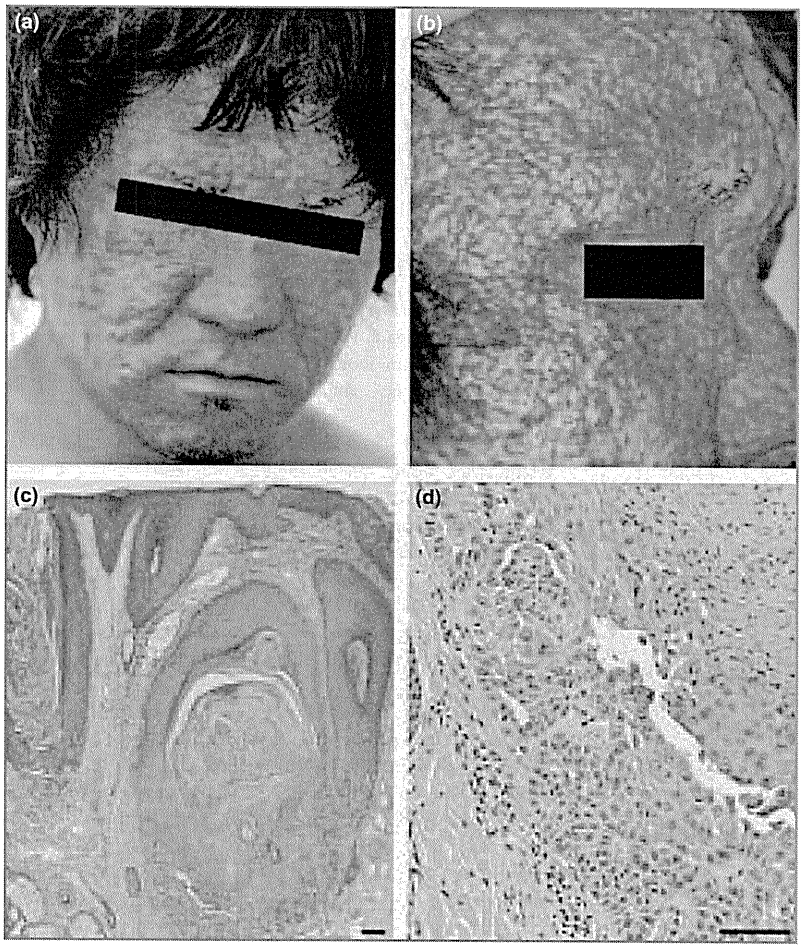


Fig 1. Unique clinical and histological features of comedonal Darier disease in the patient. (a, b) Open comedones, closed comedones, red papules, nodules, cysts and ice-pick scars are present on the face. (c, d) Histology of the comedonal lesions. Dilated, cystic hair follicles with keratin plugs are seen in the dermis (c). Suprabasal acantholytic clefts and numerous dyskeratotic cells in the form of corps ronds are apparent in the outer root sheath in the affected follicular infundibulum (d). Bars = 100 mm.

lysis,[8] a PCR product band derived from the mutant allele was amplified from the patient's genomic DNA, but not from either parent nor from the control DNA samples.

Since the first description of CDD by Derrick *et al.*,[4] only seven cases including the present one have been reported. The present case is the first in which a causative mutation has been identified. *ATP2A2* gene mutations in DD have been reported to result in alterations in calcium signalling during keratinocyte differentiation, causing acantholytic dyskeratosis.[2,9,10] The function of SERCA2 is to pump calcium from the cytosol to the endoplasmic reticulum and to excite oscillation of calcium spikes in the cytosol.[11–13] The mutation site in our patient localized to the first stalk (S1) of SERCA 2. The S1 region adjacent to transmembrane helices is considered to be highly conserved at the amino acid level by many species.[2] p.Leu41-del in S1 of SERCA2 is considered to impair calcium-binding sites in the α-helix of the region that contains a signal for sarco/endoplasmic reticulum localization, and to change the conserved alignment of five glutamic acid residues.[11,14] Several mutations in the S1 region of *ATP2A2* in DD have been reported.[2,10,14–16] In addition, the dephosphorylation process of SERCA2 is thought to be important for $Ca^{2+}$ ion release into the lumen by SERCA2 and, recently, Miyauchi *et al.*[17] reported that both p.Leu41del and p.Pro42del mutations inhibit the dephosphorylation process. The present c.120_122delGTT mutation has not been reported, although a heterozygous deletion of the identical leucine residue, c.121_123delTTA (p.Leu41del), was reported in one patient.[14] The patient showed severe hypertrophic scar formation in addition to common DD skin manifestations and had severe emotional problems and a family history of suicide.[14] Our patient with CDD had a quite different skin phenotype and showed no mental problems. We do not know exactly why the phenotype differs between our case and the previously reported cases. There is a possibility that a silent mutation or allelic variant of *ATP2A2* may have affected the phenotypic expression in our patient and/or the previously reported cases. Certain environmental factors, such as mechanical trauma, sun exposure, heat and sweating often define a phenotype of DD,[2] and such factors may be related to the formation of the CDD phenotype in our patient, because the face is more frequently affected than other body sites by environmental factors. Further functional studies are required to elucidate the pathomechanisms of CDD, a unique phenotype of DD. Interestingly, an *ATP2A2* mutation was reported to underlie two cases from one British family with another unique phenotype, acrokeratosis verruciformis (AKV), providing evidence that AKV and DD are allelic disorders.[18] On the other

 

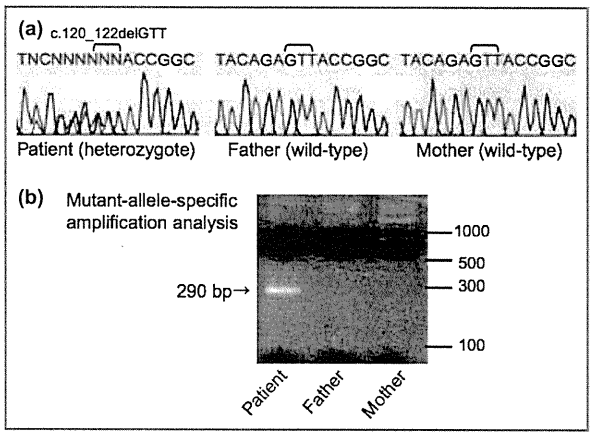


Fig 2. A heterozygous in-frame three-nucleotide deletion mutation c.120_122delGTT in *ATP2A2* was detected. (a) Direct sequencing of *ATP2A2* exon 2 polymerase chain reaction products by a reverse primer revealed that the patient was heterozygous for the three-nucleotide deletion mutation c.120_122delGTT. 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

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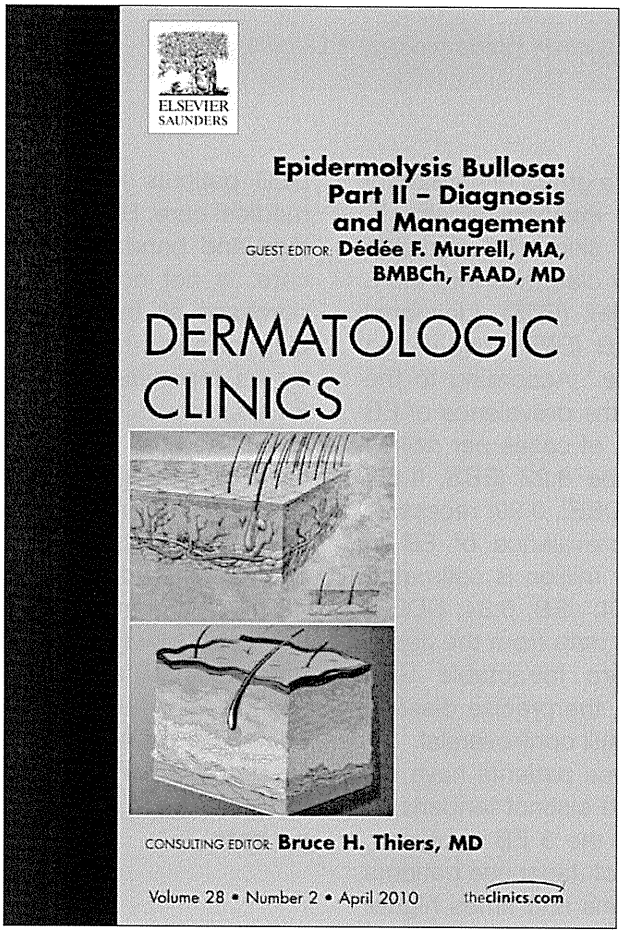

# Epidermolysis Bullosa in Japan

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Wataru Nishie, MD, PhD, Hiroshi Shimizu, MD, PhD

**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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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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**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.
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**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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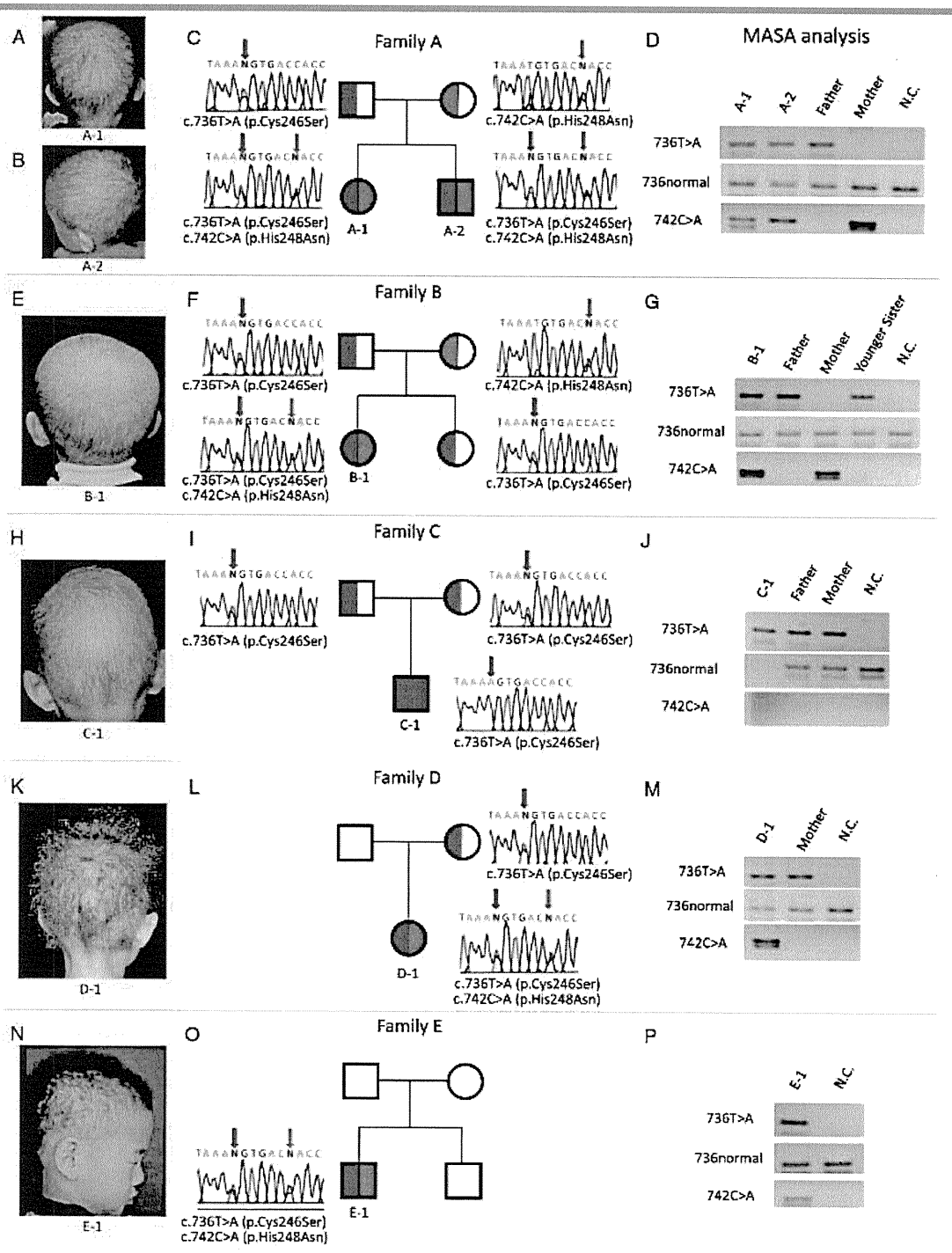



**Figure 1.** Clinical features of five Japanese families with ARH and identification of mutations in the *LIPH* gene. **A, B, E, H, K, N:** All the affected individuals have features of ARH, which is characterized by sparse hair on the scalp and slightly sparse to absent eyebrows and eyelashes. **C, 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

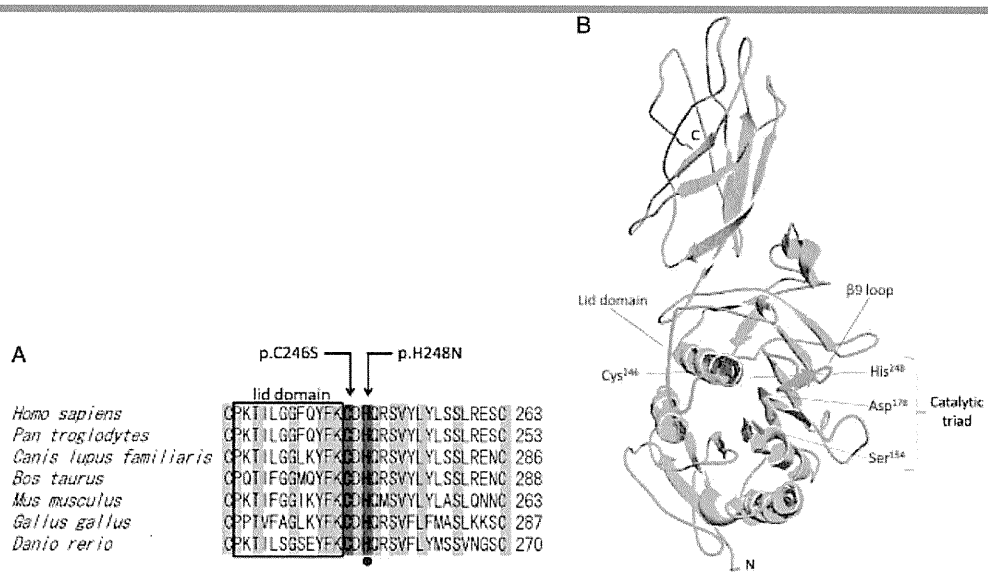
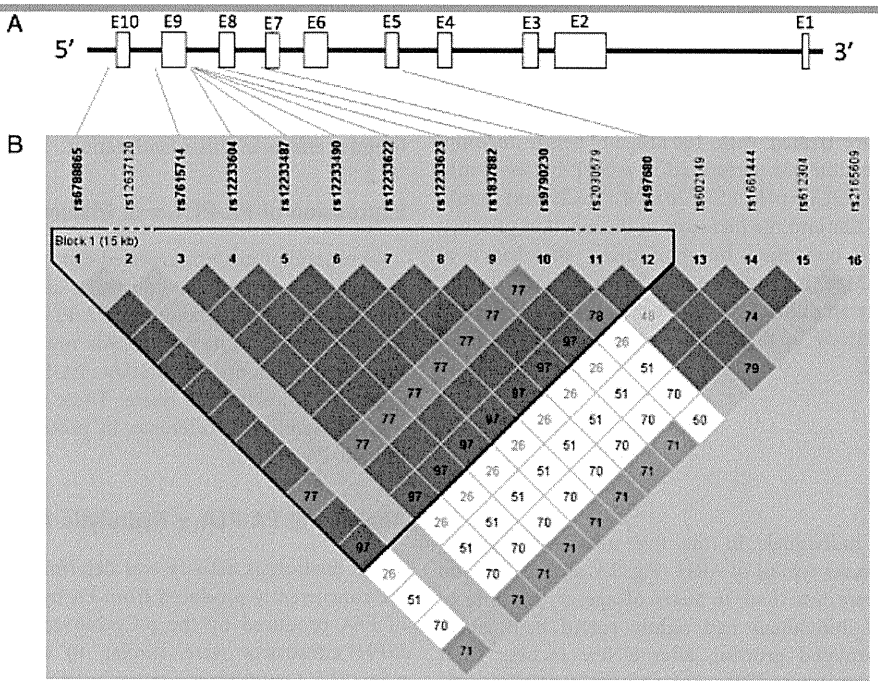



**Figure 2.** Conservation of the mutated residues and the three-dimensional protein structure around the mutation sites. **A**: Multiple amino acid sequence alignments of PA-PLA$_1\alpha$ of diverse species. Amino acid residues Cys$^{246}$ and His$^{248}$ altered by the present two mutations are highly 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.




| 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.

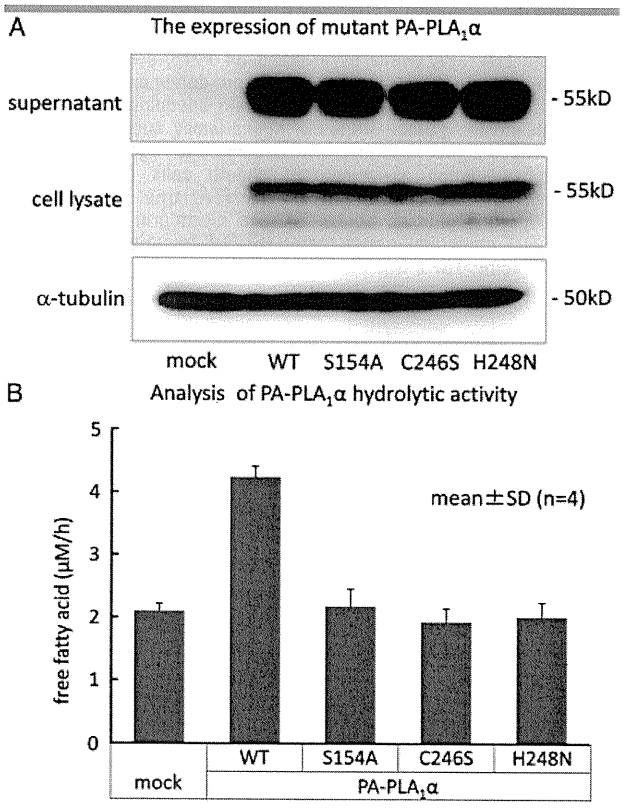



**Figure 4.** Expression of PA-PLA$_1\alpha$ in HEK293 cells and its hydrolytic activity. **A:** Expression of mutant PA-PLA$_1\alpha$ in HEK293 cells. HEK293 cells were transfected with wild-type (WT), p.Ser154Ala (S154A), p.Cys246Ser (C246S), and p.His248Asn (H248N) *LIPH* cDNA, and the expression 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.

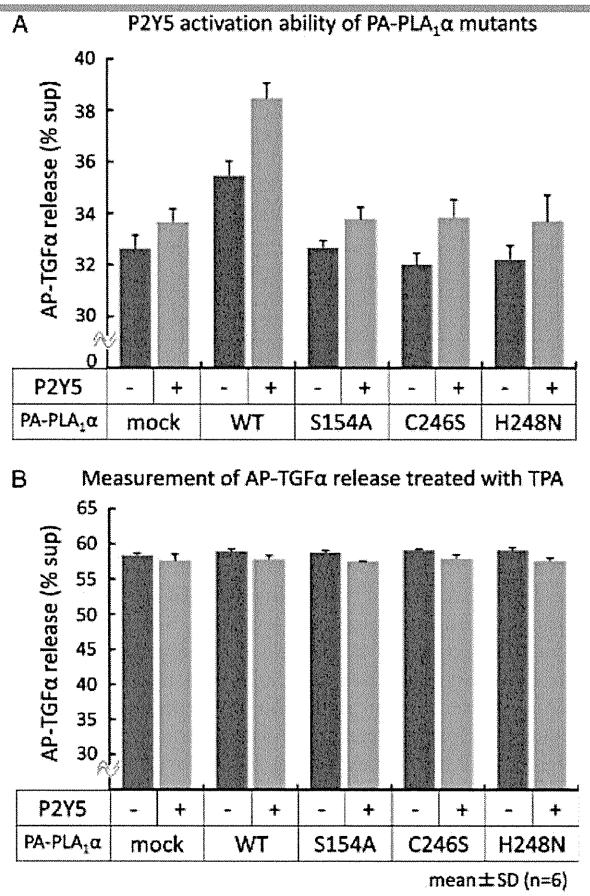



**Figure 5.** P2Y5 activation ability of PA-PLA$_1$α mutants. To monitor P2Y5 activation level by mutant and wild-type (WT) PA-PLA$_1$α, we used *p*-nitrophenyl phosphate as a substrate for cleavage of AP-TGFα and measured the amount of AP-TGFα released from the HEK293 cells. **A:** The amount of free AP-TGFα produced by P2Y5 mock-transfected (P2Y5−) cells that were also transfected with WT PA-PLA$_1$α 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$_1$α and P2Y5+ cells transfected with an empty vector (mock) are significantly lower than that from P2Y5+ cells expressing WT PA-PLA$_1$α. **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.

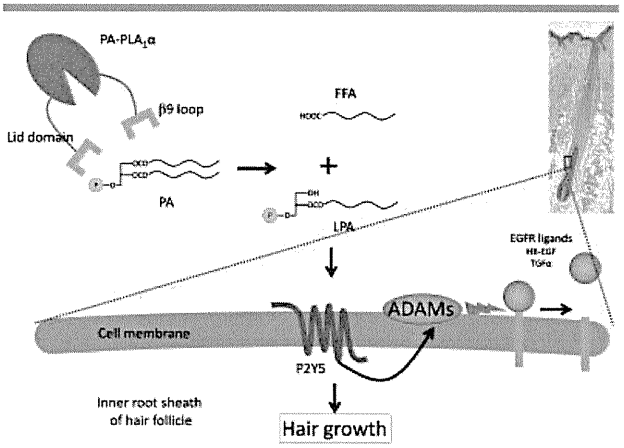



**Figure 6.** Schematic signaling pathways of LPA produced by PA-PLA$_1$α via the P2Y5 receptor. PA-PLA$_1$α hydrolyzes PA and produces LPA and FFA. LPA works as a ligand for P2Y5, a membrane-bound G-protein-coupled receptor. It has been documented that ADAM activation by P2Y5 results in ectodomain shedding 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 TGF$\alpha$ [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-TGF$\alpha$ (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

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