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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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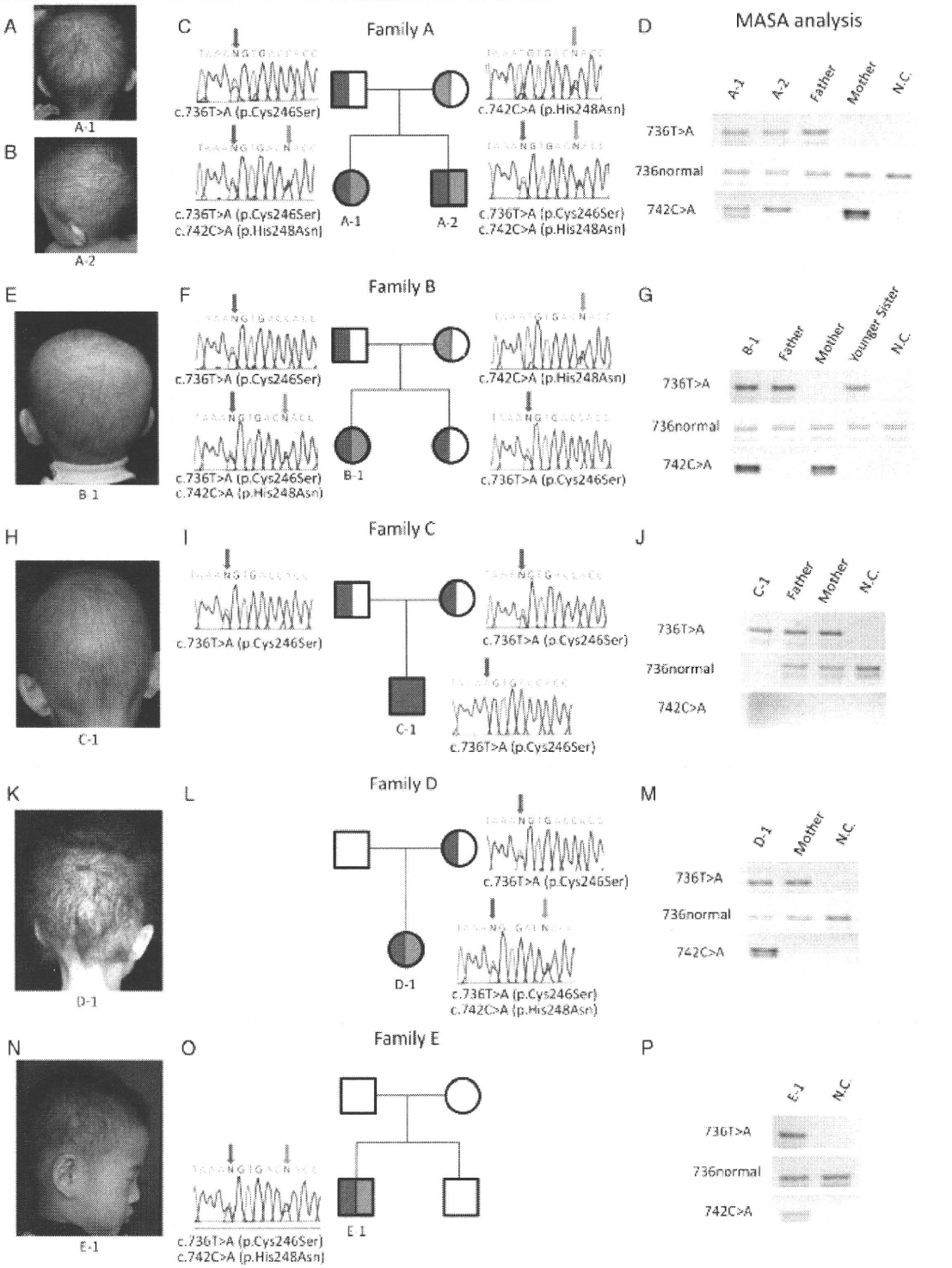


Figure 1. Clinical features of five Japanese families with ARH and identification of mutations in the *LIPH* gene. **A, B, E, H, K, N:** All the affected individuals have features of ARH, which is characterized by sparse hair on the scalp and slightly sparse to absent eyebrows and eyelashes. **C, F,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

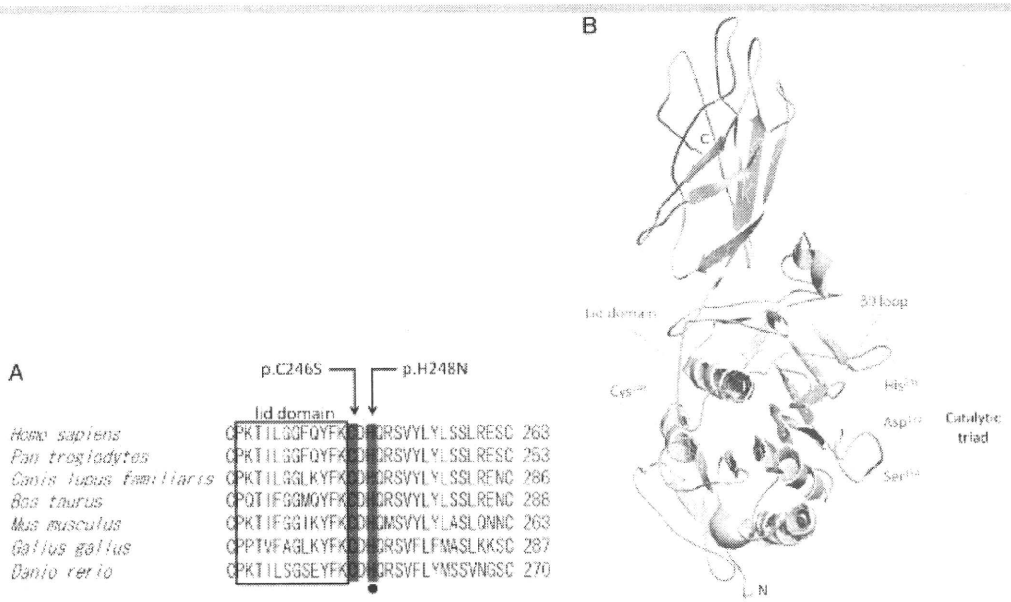
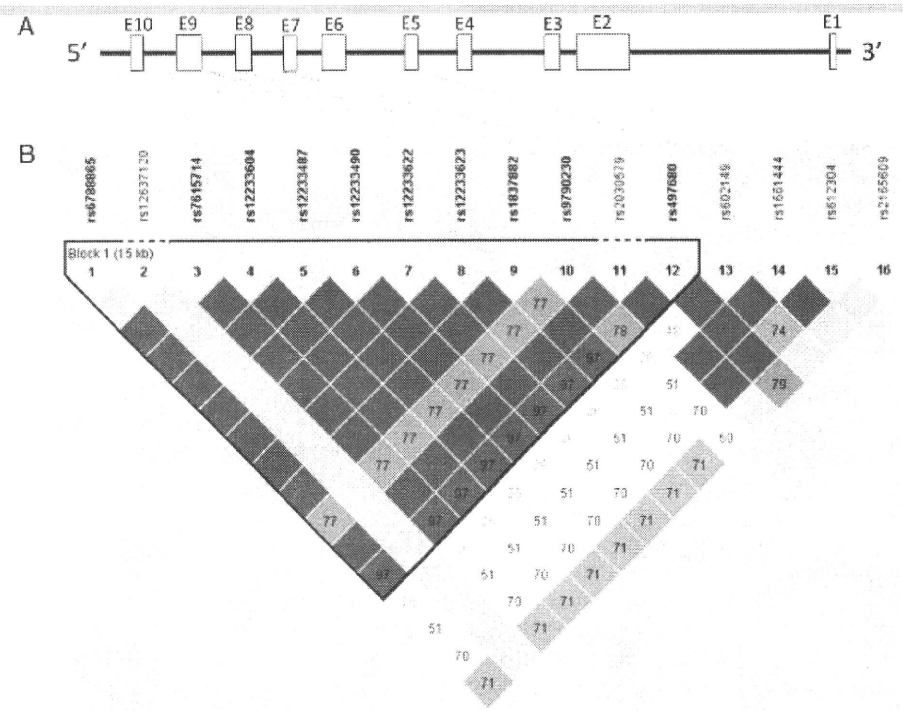


Figure 2. Conservation of the mutated residues and the three-dimensional protein structure around the mutation sites. **A**: Multiple amino acid sequence alignments of PA-PLA$_1\alpha$ of diverse species. Amino acid residues Cys^{246} and His^{248} altered by the present two mutations are highly conserved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



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.

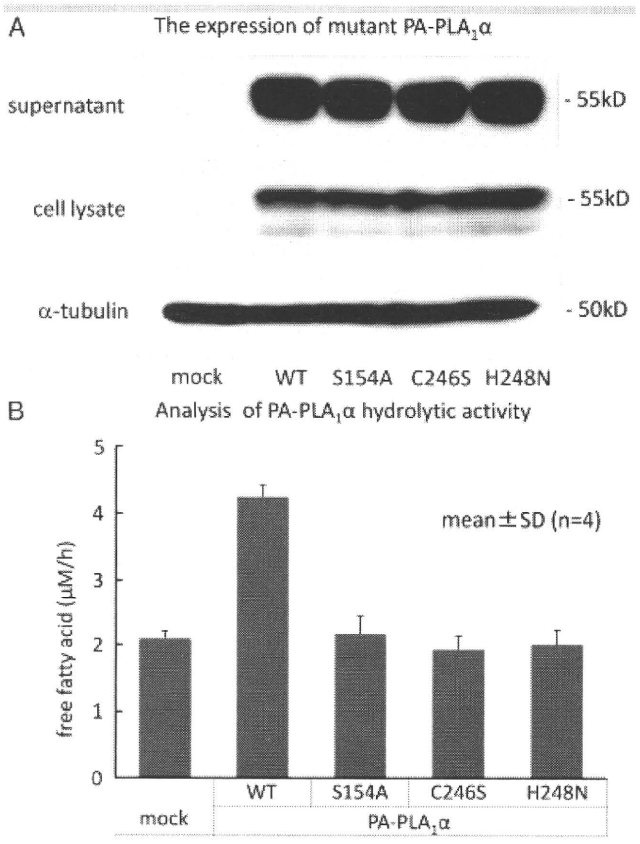


Figure 4. Expression of PA-PLA$_1\alpha$ in HEK293 cells and its hydrolytic activity. **A:** Expression of mutant PA-PLA$_1\alpha$ in HEK293 cells. HEK293 cells were transfected with wild-type (WT), p.Ser154Ala (S154A), p.Cys246Ser (C246S), and p.His248Asn (H248N) *LIPH* cDNA, and the expression level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

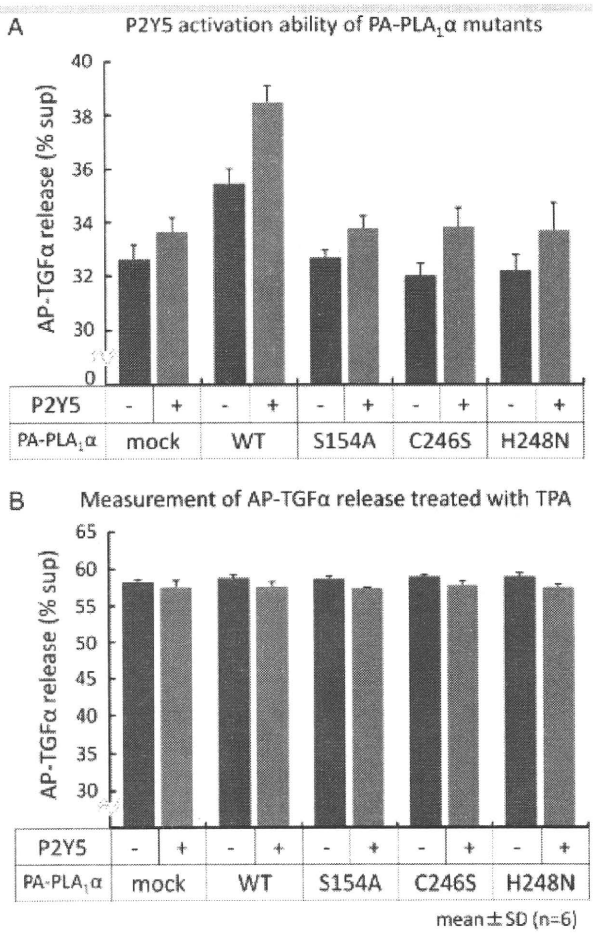


Figure 5. P2Y5 activation ability of PA-PLA$_1\alpha$ mutants. To monitor P2Y5 activation level by mutant and wild-type (WT) PA-PLA$_1\alpha$, we used *p*-nitrophenyl phosphate as a substrate for cleavage of AP-TGFα and measured the amount of AP-TGFα released from the HEK293 cells. **A:** The amount 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.

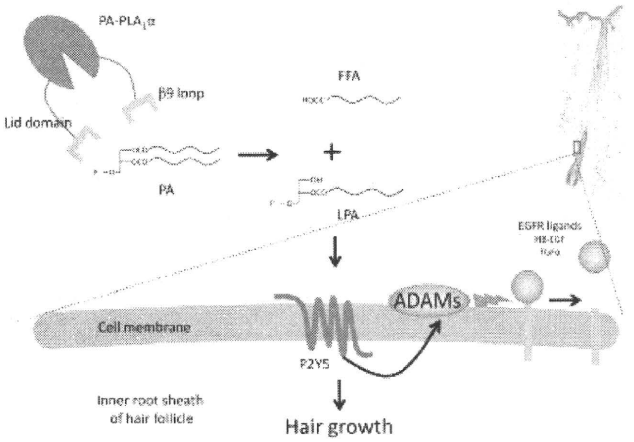


Figure 6. Schematic signaling pathways of LPA produced by PA-PLA$_1\alpha$ via the P2Y5 receptor. PA-PLA$_1\alpha$ hydrolyzes PA and produces LPA and FFA. LPA works as a ligand for P2Y5, a membrane-bound G-protein-coupled receptor. It has been documented that ADAM activation by P2Y5 results in ectodomain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

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Complete Paternal Isodisomy of Chromosome 17 in Junctional Epidermolysis Bullosa with Pyloric Atresia

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TO THE EDITOR

Uniparental disomy (UPD) is a condition in which two chromosomes of the same pair are inherited in whole or in part from only one parent. There are two types of UPD: uniparental isodisomy and uniparental heterodisomy. The former refers to two identical copies of a single homolog of a chromosome from one parent, and the latter indicates two different chromosome homologs from one parent. UPD can lead to an abnormal phenotype when isodisomy for a chromosome carrying a mutation for an autosomal-recessive disease gene results in homozygosity for the mutation.

Epidermolysis bullosa (EB) is a collection of heterogeneous disorders, in which congenital skin fragility leads to separation of the dermo-epidermal junction. EB has been subdivided into three major groups and one minor group, based on the level of blister formation: EB simplex, junctional EB, (JEB), dystrophic EB, and Kindler syndrome (Fine *et al.*, 2008). Mutations in 14 different genes have been identified as underlying EB subtypes (Fine *et al.*, 2008; Groves *et al.*, 2010). Among them, mutations in the gene encoding α6 integrin subunit (*ITGA6*) or β4 integrin subunit (*ITGB4*) are responsible for one rare subtype of JEB: JEB associated with pyloric atresia (JEB-PA). JEB-PA is inherited autosomal recessively and is characterized by generalized blistering and occlusion of the pylorus at birth, which usually leads to early demise. Most patients with JEB-PA have mutations in *ITGB4*, and more than 60 *ITGB4* mutations have been identified in JEB-PA cases.

The proband was the first child of nonconsanguineous healthy parents. There was no family history of bullous diseases. The child was born by cesarean section after a 35-week gestation because of polyhydramnion and had a birth weight of 1916 g and a birth length of 46.5 cm. Clinical manifestations of the proband included extensive blistering, especially on the extremities (Figure 1a). Routine abdominal X-ray demonstrated pyloric atresia (PA) (Figure 1b). No abnormalities other than skin fragility and PA were apparent. The proband died of sepsis 2 months after birth.

Immunofluorescence analysis revealed an absence of the β4 integrin subunit in skin specimens from the proband (Figure 1c–f). Expression of α6 integrin subunit and plectin was reduced in proband skin samples (Figure 1g and h). Immunostaining for BP230, laminin 332, and type VII collagen revealed normal linear-labeling patterns (Figure 1i–k).

Mutational analysis of all coding exons (exons 2–41) including the exon–intron boundaries of the *ITGB4* revealed that the proband was homozygous for c.953_955del in exon 8 (Figure 2a). The genomic DNA nucleotides, the

Abbreviations: EB, epidermolysis bullosa; JEB, junctional EB; JEB-PA, JEB with pyloric atresia; ITGA6, α6 integrin subunit; ITGB4, β4 integrin subunit; UPD, uniparental disomy

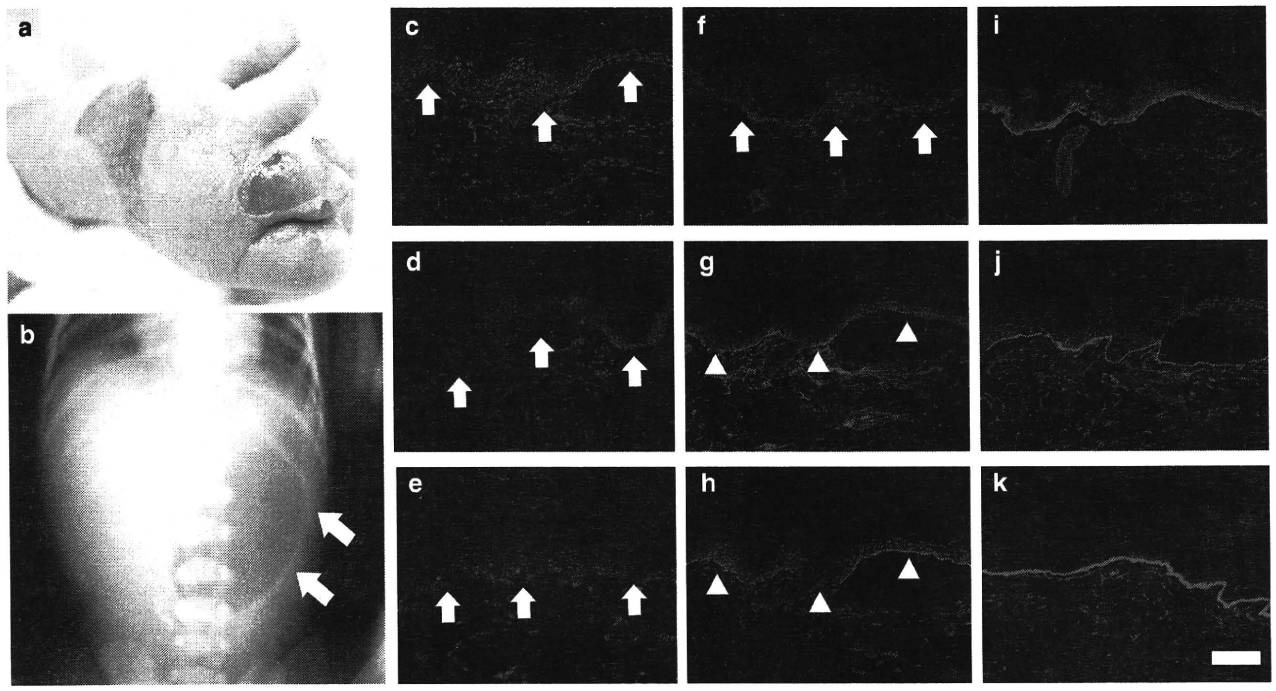


Figure 1. Clinical features of the proband and immunofluorescence analysis. (**a**) Extensive blistering is seen on the extremities at birth. (**b**) Abdominal X-ray reveals a single-bubble sign (arrows). β4 integrin subunit (3E1 (**c**), 113C (**d**), 450-9D (**e**), and 450-11A (**f**)) is not detected in the proband's skin (arrows). The expression of α6 integrin subunit (GoH3, **g**) and plectin (HD1-121, **h**) is diminished (arrowheads). BP230 (S1193, **i**), laminin-332 (GB3, **j**), and type VII collagen (LH7.2, **k**) show a normal linear staining pattern (scale bar = 100 μm). HD1-121 was donated by Professor Owaribe of Nagoya University, 113C by Professor Sonnenberg of the Netherlands Cancer Institute, 450-9D and 450-11A by Professor Lankford of the Oak Ridge National Laboratory, and S1193 by Professor Stanley of the University of Pennsylvania.

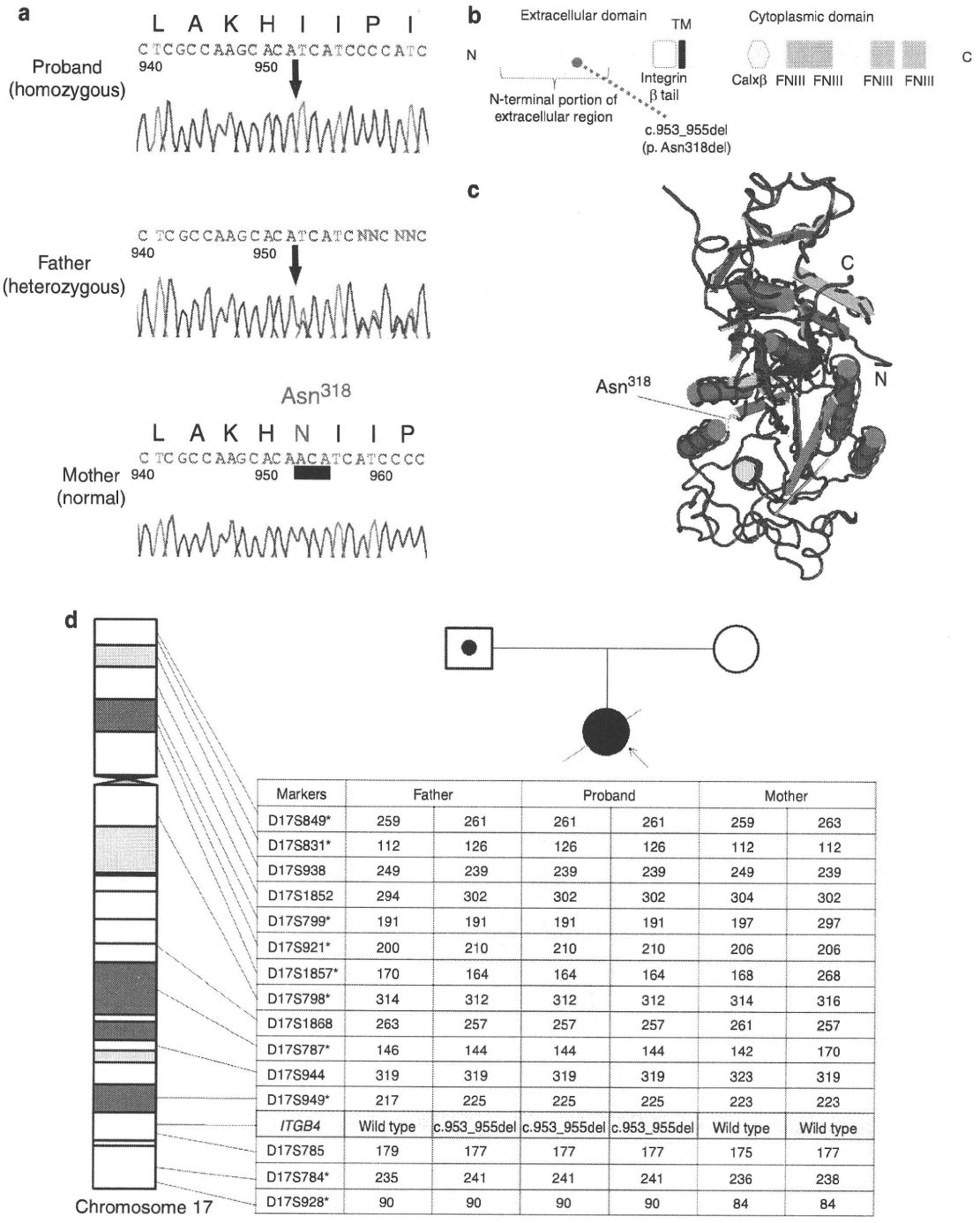
complementary DNA nucleotides and the amino acids of the protein were numbered based on the following sequence information (GenBank accession no. NM_000213). Mutation c.953_955del is predicted to result in the loss of asparagine at amino acid position 318 (p.Asn318del, Figure 2b), and this deletion mutation is not expected to cause subsequent frame-shift followed by a premature termination codon. Mutation c.953_955del was previously described in two JEB-PA cases (Iacovacci *et al.*, 2003; Varki *et al.*, 2006). The proband's father was heterozygous for this mutation (Figure 2a), although her mother revealed only normal sequences (Figure 2a).

To elucidate the origin of c.953_955del homozygosity in the proband, haplotype analysis of the entire chromosome 17 using 15 microsatellite markers (the ABI Prism Linkage Mapping Set Version 2.5 (Applied Biosystems, Warrington, UK)) was performed. The proband was found to be homozygous for all 15 microsatellite markers (Figure 2d). Ten of the 15 markers were fully informative for inheritance of two copies of a single paternal chromosome 17 in the proband (Figure 2d). For the non-chromosome 17 markers (D1S468, D1S252, D1S2842, D3S1297, D3S1566 and D3S1311), there were no discrepancies in the segregation of maternal and paternal alleles to the proband, confirming that the mother is indeed the biological mother of the patient (data not shown). Normal karyotyping ruled out monosomy of chromosome 17. The results in this family are compatible with the inheritance of two identical copies of a single chromosome 17 from the proband's father, which indicates complete paternal isodisomy of chromosome 17 in the proband. The medical ethical committee of Hokkaido University approved all the described studies. The study was conducted according to the Declaration of Helsinki Principles. Participants gave their written informed consent.

A recent review of the literature on UPD summarized 197 maternal and 68 paternal cases of UPD (Kotzot and Utermann, 2005). For UPDs of chromosome 17, only a few cases of maternal heterodisomy have been described (Genuardi *et al.*, 1999; Rio *et al.*, 2001). Recently, UPD of the whole chromosome 17 was reported as maternal heterodisomy of 17q and proximal 17p, and isodisomy of distal 17p (Lebre *et al.*, 2009). As far as we know, uniparental isodisomy of the whole chromosome 17 has not been described in the literature.

More than 35 cases of recessive-inherited disease have been reported as being caused by UPD (Kotzot and Utermann, 2005). UPD has been reported to be responsible for several EB subtypes, including Herlitz JEB (Castori *et al.*, 2008; Fassihi *et al.*, 2005; Pulkkinen *et al.*, 1997; Takizawa *et al.*, 2000; Takizawa *et al.*, 1998), EB simplex with pyloric atresia (Nakamura *et al.*, 2005) and recessive dystrophic EB (Fassihi *et al.*, 2006). So far, JEB-PA has not been described to result from UPD.

p.Asn318del (c.953_955del) has been identified as responsible for JEB-PA in two reports (Iacovacci *et al.*, 2003; Varki *et al.*, 2006). Asn^{318} in β4 integrin subunit resides in the extracellular



Markers	Father		Proband		Mother	
D17S849*	259	261	261	261	259	263
D17S831*	112	126	126	126	112	112
D17S938	249	239	239	239	249	239
D17S1852	294	302	302	302	304	302
D17S799*	191	191	191	191	197	297
D17S921*	200	210	210	210	206	206
D17S1857*	170	164	164	164	168	268
D17S798*	314	312	312	312	314	316
D17S1868	263	257	257	257	261	257
D17S787*	146	144	144	144	142	170
D17S944	319	319	319	319	323	319
D17S949*	217	225	225	225	223	223
ITGB4	Wild type	c.953_955del	c.953_955del	c.953_955del	Wild type	Wild type
D17S785	179	177	177	177	175	177
D17S784*	235	241	241	241	236	238
D17S928*	90	90	90	90	84	84

Figure 2. *ITGB4* mutation analysis. (**a**) The proband is homozygous for c.953_955 del (arrow). The proband's father is heterozygous for c.953_955 del (arrow). In contrast, the mother's gDNA shows only wild-type sequences. Deleted nucleotides are underlined. (**b**) Schematic arrangement of β4 integrin subunit and the positions of the mutation in the proband. (**c**) 3D imaging of the N-terminal extracellular domain of the integrin β chain (NCBI Conserved Domain Database (code: smart00187)) was done using Cn3D-4.1 software. Asn^{318} is in the linking loop between the α helix and the β sheet (arrow). (**d**) The proband was homozygous for all 15 microsatellite markers spanning all of chromosome 17. Ten of the 15 markers (*) suggest that the proband's alleles originated from one homolog of paternal chromosome 17.

domain of the protein (Figure 2b). This asparagine residue is an amino acid that is conserved not only in the β4 integrin subunit of vertebrates but in all the human integrin-β chains (Iacovacci *et al.*, 2003). The 3D structure of the N-terminal portion of the extracellular domain indicates that Asn^{318} consists of a linking loop between the α helix and the β sheet (Figure 2c). It is possible that the loss of this asparagine in the extracellular domain leads to significant conformational change and protein instability.

In summary, to our knowledge, we have reported the first case of complete

isodisomy of chromosome 17 and the first example of UPD underlying JEB-PA.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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CD44-Deficient Mice Do Not Exhibit Impairment of Epidermal Langerhans Cell Migration to Lymph Nodes after Epicutaneous Sensitization with Protein

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TO THE EDITOR

CD44 is a type I transmembrane protein that binds extracellular matrix nonsulfated glycosaminoglycan hyaluronan and has an important role in cell adhesion and migration (Isacke, 2002). Thus, CD44 is involved with leukocyte egress, tumor invasiveness, and metastasis (Isacke, 2002).

The role of CD44 in epidermal Langerhans cell (LC) migration to draining lymph nodes (LNs) was first evaluated by an antibody blocking system. Antibodies against CD44 epitopes inhibited emigration of LCs from the epidermis and prevented cultured LC binding to T-cell zones in LN-frozen sections (Weiss *et al.*, 1997). In a CD44-deficient mouse system, CD44 deficiency did not impair LC emigration from the epidermis, but significantly influenced their LN homing (Mummert *et al.*, 2004). In recent years, there has been significant progress in understanding the characteristics and kinetics of LCs. It is known that there are two kinds of Langerin$^+$ dendritic cells (DCs) (definition of LCs): one resides in the epidermis and another resides in the dermis (Bursch *et al.*, 2007). They show different migration patterns to draining LNs after immunization. Dermal Langerin$^+$ DC migration peaks early at 24 hours, whereas peak migration of epidermal LC is delayed until

Abbreviations: DC, dendritic cell; LC, Langerhans cell; LN, lymph node; Th, T helper

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Review

Hereditary Hearing Loss and Deafness Genes in Japan

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Hearing loss (HL) is the most common sensory impairment occurring at birth in developed countries. Epidemiological data show that more than one child in 1000 is born with HL, while more than 50% of prelingual HL cases are found to be hereditary. Approximately 70% of hereditary HL is nonsyndromic and subdivided to autosomal dominant (20%), autosomal recessive (75%), X-linked HL (1%), and maternally-inherited HL associated with the mitochondrial DNA mutation. More than 10 deafness genes have been reported to be responsible for nonsyndromic hereditary HL in Japan. Among them, the most prevalent causative genes, *GJB2* and the mitochondrial DNA *12SrRNA* are introduced. In addition, this study also refers to the specific genes responsible for the unique audiogram, mainly *WFS1*. Finally, the genes related to the enlargement of vestibular aqueduct of inner ear abnormality, *SLC26A4*, *EYA1* and *SIX1* are discussed. The clinical and genetic findings associated with these disorders including the results of a recent study are reviewed.

Key words: *GJB2, SLC26A4, 12SrRNA, EYA1, SIX1*

1. Introduction

Hearing loss (HL) is a full or partial decrease in the ability to detect or understand sounds. The types of HL include conductive HL (CHL), sensorineural HL (SNHL), and mixed HL (MHL). CHL is caused by problems in the outer and/or middle ear such as external meatal atresia and otosclerosis; SNHL is caused by problems in the inner ear, cochlear nerve, and/or central auditory pathway; and MHL is caused by a combination of both conductive and sensorineural components. The onset of HL is categorized as congenital, acquired or late-onset. Prelingual HL is either present at birth or begins before the age of five years, when language has normally been acquired. Postlingual HL occurs after the development of normal speech. The cause of HL is due to genetic (hereditary) and/or non-genetic (environmental) factors[1]. Most hereditary HL is inherited as a simple Mendelian trait and is classified into nonsyndromic or syndromic according to the presence of other disorders, such as kidney, heart, or vision abnormalities[1]. Owing to recent advances in molecular genetics, more than 130 loci and more than 40 causative genes for HL have now been identified (Van Camp G, Smith RJH. [homepage on the Internet]. Hereditary Hearing Loss Homepage, [updated 2008 May 28; cited 2009 Sep 14]. Available from: http://webh01.ua.ac.be/hhh/). This article reviews the hereditary HL and deafness genes seen particularly in the Japanese population.

2. Epidemiology of hearing loss

Epidemiological data show that HL is the most common defect at birth and the most prevalent sensory impairment in developed countries[2]. In the United

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Kingdom, where permanent childhood HL is defined as a bilateral hearing level of 40 dB or more, the incidence of congenital HL is reported to be 0.13%[3]. In the United States, 0.19% are born with SNHL of 35 dB or more in either ear, and the prevalence increases to 0.27% at 5 years and 0.35% by adolescence[4]. A recent report states that more than one of every 500 newborns has congenital HL[5]. In Japan, the prevalence of congenital HL detected by the newborn hearing screening program is calculated to be 0.08% in Okayama[6]. Although 90% of children with congenital HL have no family histories of HL, the rate of genetic contribution to HL is reported to be 68% at birth and 54% at 4 years in the United States[4,7]. Therefore, at least one child in 1000 is born with HL in developed countries and more than 50% of prelingual HL cases are found to be hereditary[8].

3. Nonsyndromic and syndromic hereditary hearing loss

More than 70% of hereditary HL is nonsyndromic[9]. Nonsyndromic hereditary HL can be subdivided to autosomal dominant, autosomal recessive, X-linked HL, and maternally-inherited HL associated with the mitochondrial DNA mutations. The loci for autosomal dominant, autosomal recessive, and X-linked nonsyndromic hereditary HL are designated as DFNA, DFNB, and DFN followed by consecutive numbers, respectively. In the nonsyndromic prelingual hereditary HL group, the manner of inheritance is 75-80% autosomal recessive, 20-25% autosomal dominant, and 1-1.5% X-linked[9]. Among 57 DFNA loci and 77 DFNB loci, 24 and 28 causative genes have been identified, respectively. Among six DFN loci, one causative gene has been found. Six different mutations in two mitochondrial genes, the *12SrRNA* and the *tRNASer*$^{(UCN)}$, can also cause nonsyndromic hereditary HL[10].

Syndromic hereditary HL accounts for up to the remaining 30%, and several hundred forms of syndromic HL are known[11]. Usher and Pendred syndromes show the representative autosomal recessive syndromic hereditary HL. The representative dominant forms of syndromic HL are Stickler, branchio-oto-renal/branchio-oto (BOR/BO), and Waardenburg syndromes[12].

4. Prevalent deafness genes in Japanese patients with hereditary hearing loss

More than 10 deafness genes have been reported to be associated with nonsyndromic hereditary HL in Japan[13]. Mutations in *GJB2*, *SLC26A4*, and the mitochondrial DNA 1555A>G mutation are the major causes of the HL[14-17].

(1) GJB2

Gap junction protein, beta-2 (*GJB2*) is the most prevalent causative gene for nonsyndromic hereditary HL in various ethnic groups[18-20]. Gap junction channels connect the cytoplasm of adjacent cells, allowing the diffusion of ions and small metabolites[21,22]. They are transmembrane proteins which are formed at the appositional plasma membranes by related proteins families named connexins[23]. *GJB2* is found to constitute gap junctions between epithelial and connective tissue cells in the cochlea and serves as the structural basis for recycling endolymphatic potassium ions that pass through the sensory and supporting cells during the sound transduction process[24].

Most of HL related with *GJB2* mutations are nonsyndromic recessive manner (DFNB1), while some mutations can cause nonsyndromic dominant hereditary HL (DFNA3)[22,25,26]. To date, more than 90 *GJB2* mutations have been reported, most of which are found in patients with moderate to profound HL[27]. Morton et al. reports that the rate of *GJB2* mutations in the United States accounts for about 21% of all HL at birth and 15% at 4 years[4]. The carrier rate for recessive deafness-causing *GJB2* mutations in the general population is approximately one in 33 in the Midwestern United States[28]. In Asian nations, *GJB2* mutations account for approximately 20% of bilateral severe-to-profound nonsyndromic HL[29-32]. In Japan, the *GJB2* mutation has been recognized in 27.5% of autosomal recessive HL[14]. The *GJB2* allelic variants are detected with a carrier rate of more than 2% among Japanese normal hearing populations[33]. Our screening (refer to the supplemental data) of nonsyndromic HL patients including 70 prelingual HL patients, 25 congenital HL patients and 46 autosomal recessive HL patients, shows that 13 (18.6%) patients with prelingual HL, 7 (28.0%) with congenital HL and 12 (26.1%) with autosomal recessive HL patients have either homozygous or compound heterozygous mutations in *GJB2*. This finding confirms that *GJB2* is the most major causative gene for nonsyndromic hereditary HL in Japan (Table I).

The prevalent genotype of *GJB2* mutations has a high ethnic predilection. By far the most commonly found mutations are deletions in two regions of *GJB2*, 35delG and 235delC[14,19]. 35delG and 235delC are the *GJB2*

Table I. Summary of Patients With biallelic *GJB2* mutation

Age/ Gender	Onset of HL	Familial History	Audiogram		Genotype	Mutation Combination
			Severity	Configuration	[allele1]+[allele2]	
26/M	3	AR	Severe	Steeply sloping	[235delC]+[235delC]	T/T
28/M	Congenital	AR	Profound	Flat	[235delC]+[235delC]	T/T
1/M	Congenital	Sporadic	Not performed		[235delC]+[235delC]	T/T
39/M	20	AR	Severe	Gently sloping	[235delC]+[235delC]	T/T
1/M	Congenital	Sporadic	Profound	Flat	[235delC]+[235delC]	T/T
40/M	3	Sporadic	Profound	Steeply sloping	[235delC]+[235delC]	T/T
13/F	Unidentified	AR	Moderate	Gently sloping	[235delC]+[G45E; Y136X]	T/T
12/F	5	AR	Moderate	Gently sloping	[235delC]+[G45E; Y136X]	T/T
30/M	Congenital	AR	Severe	Gently sloping	[235delC]+[G45E; Y136X]	T/T
5/M	3	AR	Moderate	Flat	[235delC]+[G45E; Y136X]	T/T
25/F	14	AR	Moderate	Gently sloping	[235delC]+[G45E; Y136X]	T/T
25/F	Congenital	Sporadic	Profound	Steeply sloping	[235delC]+[G45E; Y136X]	T/T
29/F	2	Sporadic	Profound	Flat	[235delC]+[504insAAGG]	T/T
26/M	Congenital	AR	Profound	Flat	[G45E; Y136X]+[G45E; Y136X]	T/T
67/F	Congenital	AR	Profound	Flat	[G45E; Y136X] + [R143W]	T/N
36/M	35	AR	Moderate	Flat	[235delC]+[V37I]	T/N
65/F	3	Sporadic	Moderate	Steeply sloping	[G45E; Y136X]+ [V37I]	T/N
10/F	7	Sporadic	Moderate	Flat	[V37I; R143W]+[R143W]	N/N
39/F	6	AR	Moderate	Gently sloping	[V37I]+[V37I]	N/N

AR: autosomal recessive, T: truncating mutation, N:non-truncating mutation

mutations detected high frequently in Caucasoid and East Asian populations, respectively. These mutations result in a frameshift and produce premature truncating protein. The large cross-sectional analyses of *GJB2* genotype-phenotype correlation data suggest that the severity of HL associated with biallelic truncating mutations is significantly more severe than that associated with biallelic non-truncating mutations[27]. Specifically, the HL with 35delG is more severe than that with non-truncating mutations including M34T, V37I, and L90P. V37I is the second most prevalent allele detected in the Japanese, followed by R143W, G45E and Y136X[14,33,34]. Homozygous 235delC has exhibited a significantly more severe phenotype than homozygous V37I and the HL with V37I has a later age onset than those with the 235delC[34]. Our study detects 235delC at the highest frequency with the rate of 20/38 (52.6%), followed by G45E, Y136X, V37I, and R143W. Homozygous 235delC causes severe-to-profound HL, while two patients associated with biallelic non-truncating mutations present moderate HL. The configuration of the audiogram is not related to either a genotype or a combination of mutations (Table I).

(2) 1555A>G mutation in the mitochondrial *12SrRNA*

12SrRNA is encoded by nucleotides 648-1601 in mitochondrial DNA, and is also called *MTRNR1*. *12SrRNA* molecules help assemble amino acids into the functioning proteins that carry out oxidative phosphorylation. The approximate prevalence of the 1555A>G mutation in the *12SrRNA* is 0.5-2.4% in European HL patients and 0.2% in European general population[35-40]. The 1555A>G mutation has also been frequently reported in various Asian countries including China and Balinese[41,42], and in Japan, we reports that 5.1% of nonsyndromic HL and 8.0% of familial HL carry the 1555A>G mutation[16]. A sequential genetic analysis shows the prevalence of this mutation to be 1.2% among the sporadic cases and 5.7% among the familial cases. Among maternally inherited HL, the prevalence of this mutation is 17.2% (refer to the supplemental data).

The 1555A>G mutation is of particular interest as a key cause of antibiotic-induced HL[43,44]. The pathogenic effect of the mutation is related to an alteration in the binding site for aminoglycosides and enlarges sensitivity to aminoglycoside ototoxicity. The antibiotic-induced HL appears dose-independently within a few days to weeks after the administration of aminoglycoside[45,46]. However, the 1555A>G mutation has been also detected in HL patients not exposed to this antibiotic[43], and other genetic and environmental factors can also influence the presence of HL caused by the 1555A>G mutation[46-48]. This mutation is found to cause a diminished ability to repair cochlear damage from a variety of causes including noise[49]. The HL in patients

Table II. Summary of Patients With A1555G mutation in the mitochondrial *12SrRNA*

Age/ Gender	Onset of HL	Familial History	Audiogram		Administration of Antibiotics	Progression of HL	Vertigo/ Dizziness
			Severity	Configuration			
37/F	6	Maternally	Severe	Gently sloping	None	-	+
44/M	Congenital	Maternally	Severe	Steeply sloping	None	-	-
39/M	10	Maternally	Mild	Gently sloping	None	+	+
37/F	20	Maternally	Moderate	Steeply sloping	None	+	+
31/F	Congenital	Maternally	Profound	Flat	None	+	+
47/F	5	AR	Mild	Steeply sloping	Streptomycin	-	-
35/F	34	AR	Moderate	Steeply sloping	None	+	-
36/F	36	AR	Severe	Steeply sloping	None	+	+
39/F	4	AR	Severe	Steeply sloping	None	+	+
75/F	37	Sporadic	Severe	Flat	Streptomycin	+	+
56/M	40	Sporadic	Severe	Steeply sloping	None	+	-
40/F	5	Sporadic	Profound	Steeply sloping	None	-	-

AR: autosomal recessive

who are not exposed to aminoglycosides starts around the age of 20 years. The penetrance of HL in patients without aminoglycoside exposure is approximately 40% by the age of 30 years, and 80% by the age of 65 years. Therefore, the 1555A>G mutation does not necessarily show a maternal inheritance pattern and the patients with this mutation can show various phenotypes ranging from completely normal hearing to profound HL. The high prevalence and low penetrance suggest that genetic analysis before the administration of aminoglycoside antibiotics would be required for subjects consanguineous with HL patients, regardless of the presence of HL. In our study, the onset of HL varies from the prelingual period to 40 years of age and the rate of patients who show a maternal familial history is lower than half. The progression of HL is recognized in 8 of 12 (66.7%) patients. Antibiotic-induced HL is observed in only two cases. The severity of HL ranges from mild to profound (Table II). The 1555A>G mutation has been found to transmit in a homoplasmic state, however, a recent report describes patients with the mutation in a heteroplasmic state[50]. Patients carrying less than 20% mutant copies are either asymptomatic or have a mild HL, whereas patients with more than 52% mutant copies have moderate to severe HL[50].

The SNHL associated with the 1555A>G mutation is found to derive from cochlear dysfunction in examinations using speech audiometry, distortion-product otoacoustic emission testing, electrocochleography and auditory brainstem responses[16]. In addition, the most severe damage to hair cells occurs toward the basal turn of the cochlea and the audiometric configuration in most cases results in high-frequency SNHL[16]. Though the vestibular dysfunction has been thought to be less common in patients with the 1555A>G mutation, our previous study shows that this mutation can cause a dysfunction of either the saccule or the inferior vestibular nerve with a preserved function of the lateral semicircular canal based on the results of caloric response testing and vestibular evoked myogenic potentials[51].

5. The deafness genes associated with low- and mid-frequency hearing loss

Although most hereditary HL causes high-frequency SNHL, some deafness genes are associated with low-frequency SNHL or mid-frequency SNHL. *DIAPH1* and *WFS1* are the representative deafness genes for low-frequency SNHL[52,53]. *TECTA* and *COL11A2* mutations can cause mid-frequency HL due to the abnormalities of the tectorial membrane in the inner ear[54,55]. The *TECTA* mutation has been reported to be identified in a Japanese family[56].

DIAPH1 is a causative gene of DFNA1 that is mapped on chromosome 5q31[52]. Bilateral HL appears in the first decade at lower frequencies than 1000 Hz, and thereafter it slowly spreads to all frequencies during adolescence, and then finally results in profound audiometric configurations.

Mutations in *WFS1* are reported to be responsible for both DFNA6/14/38 and Wolfram syndrome, which is characterized by diabetes insipidus, juvenile-onset diabetes mellitus, progressive optic atrophy and SNHL[53,57]. *WFS1* is a gene encoding an 890 amino-acid glycoprotein; wolframin, predominantly localized in the endoplasmic reticulum[57]. Heterozygous mutations in the *WFS1* can be responsible for nonsyndromic autosomal domi-

nant low-frequency SNHL (DFNA6/14/38). The majority of the mutations exist in exon 8 of the gene which encode the C-terminal domain of wolframin[58]. Either homozygous or compound heterozygous mutations can cause Wolfram syndrome[59]. The function within the inner ear is suggested to maintain calcium ion homeostasis, but the mechanism of hearing impairment mainly seen in low-frequencies in DFNA6/14/38 patients remains unclear[60].

Studies show that 75% of families affected with nonsyndromic autosomal dominant low-frequency SNHL carry the *WFS1* mutations in Europe and the United States[61]. However, the prevalence of detected mutations in Japan is much less than in Europe and the US[62,63]. Most patients with the mutation show HL in their first or second decade, and it slowly progresses with age. However, the HL does not deteriorate to become profound. Our recent studies present two novel missense mutations in Japanese, A844T and K836T[62,64] (refer to the supplemental data). The K836T in *WFS1* is identified in affected family members who show mid-frequency SNHL in childhood but gradual progression of the audiometric thresholds at lower frequencies with age[64].

6. Deafness genes associated with enlargement of the vestibular aqueduct (EVA) syndrome

(1) Enlargement of the vestibular aqueduct syndrome

The vestibular aqueduct is a narrow bony canal that opens onto the medial surface of the temporal bone and passes to the vestibule of the inner ear. A membranous tube called the endolymphatic duct runs through the vestibular aqueduct to the endolymphatic sac on the posterior surface of the petrous portion of the temporal bone, where it comes into contact with the dura mater. First described by Valvassori, an enlargement of the vestibular aqueduct (EVA) syndrome is characterized by the presence of a much larger endolymphatic duct and sac than normal and is defined on CT as a diameter greater to or equal to 1.5 mm measured midway between the operculum and the common crus[65].

EVA is the most common malformation of the inner ear associated with HL[65]. In the United States, 12% of deaf children at 4 years old are associated with EVA syndrome[4]. EVA is observed in DFNB4, Pendred syndrome, BOR/BO syndrome, distal renal tubular acidosis with SNHL, and Waardenburg syndrome[66].

(2) DFNB4/Pendred syndrome

Pendred syndrome is an autosomal recessive disorder usually characterized by progressive severe-to-profound bilateral SNHL, vestibular dysfunction, EVA and development of euthyroid goiter[67]. DFNB4 was initially described in a Middle-Eastern Druze family with recessive nonsyndromic deafness and has been now recognized as nonsyndromic SNHL associated with EVA[15,68]. DFNB4 and Pendred syndrome comprise a phenotypic spectrum of HL either with or without thyroid defects[68-70]. Patients with DFNB4/Pendred syndrome may be unusually vulnerable to inner ear disease associated with head injury. Presumably this vulnerability occurs because there is an increased compliance of pressure waves in the brain to the inner ear.

The gene responsible for DFNB4/Pendred syndrome is known to be *SLC26A4*[71]. *SLC26A4* is mapped on 7q31, belonging to the sulfate ion transporter, and encodes a 780-amino acid (86-kD) protein known as pendrin[69]. Pendrin seems to be responsible for the efflux of iodide in thyrocytes, and for mediating $Cl^-/HCO3^-$ exchange in inner ear[72-74]. Pendrin regulates the pH of endolymphatic fluid by $HCO3^-$ secretion, and modifies inner ear acid-base homeostasis[75].

A sequence analysis of HL associated with EVA, except patients inherited as an autosomal dominant, has identified disease-causing mutations in approximately 80% of familial cases and in 20% of sporadic cases in the United States[71]. A study of 274 East Asians and 318 South Asians with SNHL demonstrates that mutations in *SLC26A4* are recognized in approximately 5% of both groups[76]. Furthermore, an analysis in Japan shows that *SLC26A4* mutations are responsible for 90% of families with Pendred syndrome, and for 78% of nonsyndromic familial HL with EVA[77]. Three recurrent mutations in *SLC26A4*, being L236P, T416P, and IVS8+1G>A, account for approximately 50% of the variant alleles detected in DFNB4/Pendred syndrome in Caucasians of northern European descent[71,78]. In East Asia, the spectrum of *SLC26A4* mutations in the Chinese population reveals that IVS7-2A>G is the most frequent mutation which accounts for about half, while the most prevalent mutation in Korea is H723R with a rate of 40%[79,80]. In Japan, H723R is the predominant mutation and accounts for more than 50% of deafness-causing *SLC26A4* mutations[15,77,81]. The reason for the high prevalence of H723R is considered to be due to the founder effect rather than due to a mutational hot spot[76].

In our recent study, 10 patients with SNHL and EVA are screened for *SLC26A4* mutation, excluding patients with dominant types of HL (refer to the supplemental data). Their phenotype shows Pendred syndrome in

Table Ⅲ. Clinical features and analysis of *SLC26A4 for* the 10 subjects with EVA

Age/ Gender	Onset of HL	Severity of HL	Progression of HL	Vertigo/ Dizziness	Familial History	Phenotype	Genotype [allele1] + [allele2]
8/F	7	Moderate			Sporadic	Nonsyndromic	
3/M	1	Severe			AR	Nonsyndromic	[H723R]+[-]
11/F	3	Severe	+	+	Sporadic	Nonsyndromic	[H723R] + [H723R]
24/F	22	Mild	-	+	Sporadic	Nonsyndromic	
24/F	Congenital	Profound	-	+	Sporadic	Pendred	[H723R] + [T410M]
23/F	6	Severe	+	-	Sporadic	Nonsyndromic	
28/F	1	Profound	+	+	AR	Nonsyndromic	
26/F	2	Profound	+	+	Sporadic	Pendred	[H723R] + [IVS15+5G>A]
29/F	2	Profound	+	+	AR	Pendred	[H723R] + [H723R]
24/F	1	Profound	+	+	AR	Pendred	[IVS5+1G>T] + [G439R]

AR: autosomal recessive

four patients and nonsyndromic HL in six patients. Mutations are identified in all four Pendred patients (100%) and one of 6 patients showing nonsyndromic HL (16.7%). The detected mutations include three missense and two splice site variants. H723R is the most common with a prevalence of 60% and a novel mutation, IVS5+1G>T is detected in that study. All patients carrying a biallelic *SLC26A4* mutation show prelingual sever-to-profound HL and disequilibrium symptoms and 4/5 (80%) show progressive HL (Table III). The previous other report about the *SLC26A4* mutation in Japan, similarly, shows that approximately 90% show progressive HL and 70% complain of vertigo[81]. The goiterous phenotype in Pendred syndrome is not recognized in childhood and is found to develop with age[81]. Therefore, an 11-year-old nonsyndromic female patient with homozygous H723R may be categorized as Pendred syndrome (Table III).

(3) BOR/BO syndrome

BOR syndrome is an autosomal dominant developmental disorder characterized by HL, anomalies of branchial arch system and renal malformations. It has an estimated prevalence of 1:40000 and 2% of profoundly deaf children are affected with BOR syndrome[82]. HL is found in more than 90% of patients affected with BOR syndrome. The severity of HL can be from mild to profound in affected individuals in the same family. The type of HL is MHL (50%), CHL (25%), and SNHL (25%) and the progression and fluctuation of HL does not always occur[82]. The severity seems to be predisposed to progress due to EVA[83,84]. The branchial anomaly includes branchial fistulae and cysts, preauricular pits, pinnae deformities and external auditory canal stenosis. The presence and combination of otologic, audiological and branchial arch abnormalities may thus show extreme variation. Renal malformations are not infrequent and they range from mild hypoplasia to a complete absence[82,85]. This syndrome can be called BO syndrome, when occurring without any renal malformation.

Three genes, *EYA1*, *SIX5* and *SIX1*, are known to be associated with BOR/BO syndrome. The embryonic development of the ear depends on the *EYA- SIX* hierarchy of regulatory genes[86]. *EYA1* consists of 16 coding exons that extend over 156 kb, including a highly conserved particular structure in the *Eya* gene family known as the eya-homologous region (eyaHR)[87]. The eyaHR mediates interactions with the gene products of *so* in Drosophila. The vertebrate orthologues of *so* are members of the *Six* gene family[88]. The *SIX1* has two exons coding for a transcript of 1376 bp. *SIX1* has a conserved SIX domain interacting of *EYA1*, and a homeodomain requiring for DNA binding. The normal expression of both *EYA1* and *SIX1* are necessary for appropriate development of the middle and inner ear[86,89]. The *SIX5* has three exons of a transcript of 3145 bp, having 739 amino acid residues. *SIX5* demonstrates a high degree of homology to *SIX1* and it is also known to interact directly with *EYA1*[90].

The *EYA1* mutations are detected in approximately 40% of patients with BOR/BO syndrome[91]. The *SIX5* mutations are detected in 5.2% of patients with BOR/BO syndrome without *EYA1* mutation[92]. *SIX1* mutations are thought to account for only a small proportion of patients with BOR/BO syndrome[90]. In our study, five patients with BOR/BO syndrome are screened for the *EYA1* and *SIX1* mutation (refer to the supplemental data). The *SIX1* and *EYA1* mutations are detected in two and three patients, respectively (Table IV)[66,93]. These findings suggest that BOR/BO syndrome caused by *SIX1*mutation is not so rare in Japanese and tends to lack apparent branchial anomalies, and thus may be