Analysis of unique mutations in the LPAR6 gene identified in a Japanese family with autosomal recessive woolly hair/hypotrichosis: Establishment of a useful assay system for LPA6

Ryota Hayashi a,b, Asuka Inoue c,d, Yasushi Suga e, Junken Aoki c,f, Yutaka Shimomura a,b,*

a Laboratory of Genetic Skin Diseases, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan
b Division of Dermatology, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan
c Laboratory of Molecular and Cellular Biochemistry, Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai, Japan
d PRESTO, Japan Science and Technology Agency (JST), Kawaguchi, Japan
e Department of Dermatology, Juntendo University Urayasu Hospital, Urayasu, Japan
f CREST, JST, Kawaguchi, Japan

A R T I C L E  I N F O

Article history:
Received 11 December 2014
Received in revised form 14 February 2015
Accepted 4 March 2015

Keywords:
Woolly hair
Hypotrichosis
LPAR6
LPA6
LIPH

A B S T R A C T

Background: Woolly hair (WH) is a hair shaft anomaly characterized by tightly-curved hair and is frequently associated with hypotrichosis. Non-syndromic forms of WH can show either autosomal dominant or recessive inheritance. The autosomal recessive form of WH (ARWH) is caused by mutations in either lipase H (LIPH) or lysophosphatidic acid receptor 6 (LPAR6) gene, encoding an LPA-producing enzyme PA-PLA2α and an LPA receptor LPA6, respectively.

Objective: To define the molecular basis of ARWH/hypotrichosis in a Japanese family.

Methods: We performed mutational analysis of candidate genes and a series of expression and in vitro functional analyses, which we improved in this study, to determine the consequences resulting from the mutations identified in the family.

Results: Novel compound heterozygous LPAR6 mutations were identified in the patient. One was a nonsense mutation c.756T>A (p.Tyr252*); the other was a large insertion mutation within the promoter region of LPAR6. Expression studies detected LPAR6 mRNA only from the c.756T>A allele in the patient’s hair follicles, suggesting that the insertion in the other allele disrupted the LPAR6 promoter and thus led to a failure of transcription. Furthermore, an improved LPA6 functional assay developed in this study demonstrated aberrant expression and a subsequent loss of function of the p.Tyr252* -mutant protein.

Conclusion: Through establishing a useful assay system for LPA6, our results further underscore the crucial roles of LPAR6 in hair follicle development and hair growth in humans at molecular levels.

© 2015 Japanese Society for Investigative Dermatology. Published by Elsevier Ireland Ltd. All rights reserved.

1. Introduction

Woolly hair (WH) is a hair shaft anomaly that is characterized by short, thin, and tightly curled hair. Scalp hairs of affected individuals with WH typically stop growing at a few inches. In addition, hair loss can progress gradually with aging and result in hypotrichosis. Therefore, WH is considered to be a kind of hair growth deficiency [1]. WH is generally classified into syndromic and non-syndromic forms. In the syndromic forms of WH, affected individuals show not only hair symptoms, but also various cutaneous and non-cutaneous complications, such as palmoplantar keratoderma, bone anomaly, and heart failure [2]. By contrast, WH is the only main symptom in the non-syndromic forms, although other cutaneous findings (such as dry skin and keratosi pilaris) can appear in some cases [3]. The non-syndromic forms of WH show either an autosomal dominant (ADWH; OMIM 194300) or autosomal recessive (ARWH; OMIM 278150/604379) inheritance [1]. ADWH results

Abbreviations: WH, woolly hair; AD, autosomal dominant; AR, autosomal recessive; IRS, inner root sheath; HF, hair follicle; LPA, lysophosphatidic acid; GPCR, G protein-coupled receptor; PCR, polymerase chain reaction; RT, reverse transcription; AP, alkaline phosphatase; WT, wild-type; p-NPP, p-nitrophenylphosphate.

* Corresponding author at: Laboratory of Genetic Skin Diseases, Niigata University Graduate School of Medical and Dental Sciences, 1-757 Asahimachi-dori, Chuo-ku, Niigata 951-8510, Japan. Tel.: +81 25 227 2025; fax: +81 25 227 0393.
E-mail address: yshimo@med.niigata-u.ac.jp (Y. Shimomura).

http://dx.doi.org/10.1016/j.jdermsci.2015.03.006
0923-1811 © 2015 Japanese Society for Investigative Dermatology. Published by Elsevier Ireland Ltd. All rights reserved.
from mutations in either keratin 74 (KRT74) or keratin 71 (KRT71); each of these genes encodes a type II epithelial keratin and is specifically expressed in the inner root sheath (IRS) of hair follicles (HFs) [4,5]. Similarly, two causative genes for ARWH have been identified: lipase H (LIPH) and lysophosphatidic acid receptor 6 (LPAR6) [6–9].

LIPH encodes a phosphatidic acid-selective phospholipase A₁ (PA-PLA₁)α that is localized at the outer layer of the plasma membrane and produces 2-acyl-lysophosphatidic acid (LPA) from phosphatidic acid [10]. LPAR6, previously named P2RYS, is a nested gene that is located within intron 17 of retinoblastoma 1 (RB1) on chromosome 13q14 [11]. LPAR6 comprises a single coding exon and encodes a G protein-coupled receptor (GPRC) LPA₆, which is also called P2YS and is an LPA receptor [8,12]. Importantly, expression patterns of PA-PLA₁ and LPA₆ overlap in the IRS of HFs, and thus the lipid mediator is believed to play a crucial role in HF development and hair growth in humans [5,7,9]. Furthermore, we (A.I. and J. A.) have reported that Liph-knockout mice show a wavy coat phenotype that is similar to WH in humans [13]. Detailed analysis of this mouse model, together with functional studies in cultured cells, demonstrate that PA-PLA₁/LPA₆ signaling activates tumor necrosis factor-α-converting enzyme (TACE), thereby inducing ectodermal shedding of transforming growth factor-α (TGFα) and causing transcriptional inhibition of epidermal growth factor receptor (EGFR). Using this phenomenon, an efficient assay system for GPCR-mediated signaling has also been established, while detection of LPA₆ activation was not so much sensitive [13,14].

To date, numerous distinct mutations in both LIPH and LPAR6 have been reported to be causative for ARWH/hypotrichosis in several human populations [6–9,15–37]. In the Japanese population, however, only LIPH mutations have been identified in families with the disease, while LPAR6 mutations have not yet been found in any Japanese patient [19,22,23,25,26]. We herein describe a Japanese family with ARWH/hypotrichosis, the identification of unique compound heterozygous mutations in LPAR6 in this patient, and the establishment of an assay system to sensitively analyze the function of LPA₆.

2. Materials and methods

2.1. Subjects

The patient was a 36-year-old male of Japanese origin (II-3 in Fig. 1A). He has had tightly curled scalp hairs since birth (Fig. 1B). His eyebrows, eyelashes, and beard hairs looked normal. With aging, loss of scalp hairs has progressed and led to an obvious hypotrichosis phenotype (Fig. 1C). When he visited our hospital, he had his scalp hairs shaved (Fig. 1D). Interestingly, several patchy alopecia lesions were evident on his scalp skin (Fig. 1D). However, we could not completely exclude the possibility of alopecia areata because we were unable to obtain permission for a skin biopsy. Besides the hair symptoms, he did not show any other associated findings, such as atopic dermatitis, keratosis pilaris, dystrophic nails, palmoplantar keratoderma, or bone anomaly. One of his elder brothers also showed similar hair abnormalities (II-2 in Fig. 1A; data not shown). There were no consanguinities between the parents, who were unaffected and had straight scalp hairs with normal density.

2.2. Mutational analysis of LIPH and LPAR6

After obtaining informed consent, we collected peripheral blood samples or plucked hairs from the family members and 100 population-matched unrelated healthy control individuals (under institutional approval and in adherence to the Declaration of Helsinki Principles). Sampling from one sibling (II-2 in Fig. 1A) was unavailable. Standard techniques were used to isolate genomic DNA from each sample. Genomic DNA samples from the family members were used for direct sequencing analysis of the coding regions of LIPH and LPAR6; gene-specific primers and polymerase chain reaction (PCR) conditions described previously were used [7,9]. To screen for the mutation c.756T>A (p.Tyr252*) in LPAR6, PCR was performed with a forward primer (LPAR6-ORF-F2: 5'-CCAGAAGCCACATGGAAAAC-3') and a reverse primer (LPAR6-ORF-R2: 5'-CAGCAATACAGAGAGTGATTGG-3'). The amplified products were digested with PsiI restriction enzyme at 37 °C for 3 h, and the reaction products were run on 7.0% polyacrylamide gels.

In order to search for other mutations in LPAR6, a part of the LPAR6 promoter sequences was PCR-amplified with the forward primer (LPAR6-P-F1: 5'-TGTCACCATAGGTTGATATC-3') and the reverse primer (LPAR6-P-R1: 5'-TGACAGCTCTTATCCGTGGTG-3'); the amplification products were analyzed on 0.8% agarose gels. Subsequently, a PCR product from the patient's DNA was cloned into the pCRII-TOPO vector (Life Technologies, Carlsbad, CA, USA) via TA-cloning, and the positive clones were analyzed by direct sequencing. To screen for the insertion mutation in the LPAR6 promoter, PCR was performed using an insertion-specific forward primer (LPAR6-ins-F: 5'-GTTCTTCTTAGTCTGAGGTC-3') and an LPAR6-specific reverse primer (LPAR6-JUT-R: 5'-GACACTTTTTCA-CAGTTGAAGAAC-3'); all amplification products were analyzed on 0.8% agarose gels. In addition, a part of intron 23 of the RB1 gene

Fig. 1. Family pedigree and clinical appearance of the patient. (A) Pedigree of the family. The affected individual analyzed in this study (II-3) is indicated by an arrow. (B–D) Clinical features of the affected individual (II-3) at the age of 1 (B), 13 (C), and 36 (D). Notably, he had obvious woolly scalp hairs when he was a child (B and C).
was PCR-amplified with the forward primer (RB1-F: 5′-GAAGGCC-CATCAGACTAAGCC-3′) and the reverse primer (RB1-R: 5′-TCATTCTCCGTATCCACACGC-3′). The PCR products were run on 0.7% agarose gels.

2.3. Reverse transcription (RT-PCR)

With plucked hairs collected from the patient and a healthy Japanese control individual, total RNA was isolated using the RNeasy® Minikit (Qiagen Inc., Valencia, CA, USA). To completely remove genomic DNA, RNA samples were each digested with RNase-free DNase I (Takara Bio Inc., Tokyo, Japan) at 37°C for 1 h; these reactions were followed by reverse transcription using oligo-dT primers and the SuperScript III reverse transcriptase (Life Technologies). The first strand cDNA were used as templates to PCR amplify cDNAs of LPAR6 and RB1. The primer pair for LPAR6 comprised LPAR6-ORF-F2 and LPAR6-ORF-R2. The RB1-cDNA was amplified with the forward primer (RB1-F: 5′-TTGGCTGGCCTTGACGTT-3′) and the reverse primer (RB1-R: 5′-GGCTGCTGTACATACCATCCTG-3′), which were complementary to sequences in RB1 exons 16 and 25, respectively. The PCR products were run on 1.0% agarose gels, and were also directly sequenced.

2.4. Generation of expression vectors

Expression vectors encoding alkaline phosphatase (AP)-conjugated TGFα and wild-type (WT) or p.Ser154Ala (S154A)-mutant human PA-PLA2-A were generated previously [13]. Note that codons within the LPA6-expression construct were optimized (Supplementary Fig. 1) and synthesized by Genscript (Piscataway, NJ, USA) so that the amino acid sequences were unchanged and the efficiency of membrane localization of recombinant LPA6 was maximized. A Flag epitope tag (DYKDDDDK) was inserted between the first and the second codons. An expression construct encoding the p.Tyr252* (Y252X)-mutant LPA6 was generated by introducing a stop codon (TAA) at p.Tyr252 of the codon-optimized WT LPA6 expression construct; the TAT in the codon-optimized template sequence (Supplementary Fig. 1) was mutated to TAA in the p.Tyr252* (Y252X)-mutant LPA6 construct.

2.5. Flow cytometric analysis

HEK293FT (human embryonic kidney) cells were seeded on a 12-well plate (2 × 10^5 cells per well) one day before transfection. Each expression construct encoding a recombinant LPA6 (WT, 1 to 100 ng per well; Y252X, 100 ng per well) or an empty vector (100 ng per well) were transfected into separate populations of cells with Lipofectamine 2000 (Life Technologies). Approximately 24 h after transfection, cells were harvested with 2% goat serum-containing PBS, labeled with anti-FLAG mAb (clone 2H8, 10 μg/mL; Transgenic Inc., Japan), and then labeled with Alexa 488-conjugated anti-mouse IgG (10 μg/mL; Life Technologies). Fluorescent signal was measured with an SH800 flow cytometer (Sony, Tokyo, Japan).

2.6. TGFα shedding assay

TGFα-shedding assays were performed as described previously [14]. Briefly, HEK293FT cells seeded on a 12-well plate were co-transfected with an AP-TGFα-expression construct and an expression construct encoding LPA6 (WT, 0.1 to 100 ng per well; Y252X-mutant, 100 ng per well) or an empty vector (100 ng per well). Approximately 24 h after transfection, cells were harvested, suspended in 3 mL HBSS, and re-seeded into a 96-well plate (80 μL per well). Cells were then treated with an LPA6-3 antagonist Ki16425 (10 μL per well; final concentration of 10 μM) and stimulated with 1-alkyl-OMPT (10 μL per well; final concentration ranging from 100 pM to 1 μM) or LPA (10 μL per well; final concentration ranging from 1 nM to 3.2 μM) for 1 h. The metabolically stable analogue of LPA (1-alkyl-OMPT) potently activates LPA4 [38]. Samples of conditioned media (80 μL per well) were transferred into respective wells of another 96-well plate; for each sample of conditioned media and the cell surfaces in the original wells, p-nitrophenylphosphate (p-NPP) solution was added to each well, and AP activity was determined by measuring optical absorbance at 405 nm. AP-TGFα release was calculated from a percentage of AP activity in conditioned media followed by subtraction of a baseline (spontaneous) AP-TGFα release response. GraphPad Prism 6 software (GraphPad) was used to fit each concentration–response curves to a respective four-parameter sigmoid mode and determine the associated E_{max} and EC_{50} Values.

In another experiment designed to monitor PA-PLA2-α-mediated LPA6 activation, HEK293FT cells were suspended in Opti-MEM (Life Technologies), seeded in a 96-well plate (80 μL per well), and mixed with a transfection solution (20 μL per well) containing Lipofectamine 2000 and one of the follow combinations of plasmids: (1) AP-TGFα-expression vector (20 ng per well), (2) LPA6-expression vectors (WT, 0.08 to 8 ng per well; Y252X, 8 ng per well) or an empty vector (Mock, 8 ng per well) and (3) PA-PLA2-α-expressing vectors (WT or catalytically inactive mutant (S154A), 0 to 0.5 ng per well). The cells were cultured for 24 h after the transfection.

Samples of conditioned media (80 μL per well) from the cultured cells were transferred into respective wells of a 96-well plate, and AP activity in the conditioned media and cell surface was quantified by adding p-NPP solution and measuring optical absorbance at 405 nm as described previously [13,39].

3. Results

3.1. Identification of a heterozygous nonsense mutation in the LPAR6 gene

Based on clinical features, we diagnosed our patient as having ARWH/hypotrichosis and performed direct sequencing analysis of the LIPH and LPAR6 genes. Although we did not find mutations in the LIPH gene, we identified a heterozygous nonsense LPAR6 mutation c.756T > A (p.Tyr252*) in the patient (Fig. 2A). Screening assay with the PsiI restriction enzyme showed that the patient’s mother (I-2) was also heterozygous for this mutation, while the other unaffected family members and 100 Japanese control individuals did not carry it (Fig. 2B; data not shown). We did not find any other sequence variants within the coding or 5′- and 3′-noncoding regions (data not shown) of LPAR6 in the patient.

3.2. Identification of a heterozygous insertion in the LPAR6 promoter region

Because the unaffected mother of the patient carried the c.756T > A mutation in a heterozygous state, we reasoned that the patient’s other allele might have a mutation in LPAR6 promoter region. To test this hypothesis, we designed a series of primers to analyze the LPAR6 promoter and performed PCR amplification with these primers and genomic DNA templates. To our surprise, PCR with one primer pair (LPAR6-P-F1 and LPAR6-P-R1; Fig. 2C) amplified not only the expected product of 851 bp from the patient’s DNA, but also an extraordinarily large product that was approximately 5.0 kb in size (Fig. 2D). We cloned this large product into the pCRII-TOPO vector and analyzed the sequence of a positive clone. The results demonstrated that these LPAR6 sequences contained a 4156 bp-insertion in the LPAR6-promoter region (Fig. 2C). The insertion occurred 248 bp upstream from the LPAR6
transcription start site, and there was a 14 bp-duplication of LPAR6 promoter sequences at both borders of the insertion (Fig. 2C). We then performed screening assay using an insertion-specific forward primer (LPAR6-ins-F) and an LPAR6-specific reverse primer (LPAR6-3UTR-R) and found that both his unaffected father (I-1) and brother (II-1) also had this insertion (Fig. 2E). Neither his mother (I-2) nor 100 control individuals carried this insertion (Fig. 2E; data not shown). Direct-sequencing analysis of the PCR product from the patient showed WT sequence at position c.756 in the LPAR6 gene (Fig. 2F). Detailed analysis of the insertion using NCBI-BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) revealed that the sequences were derived from intron 23 of the RB1 gene (Supplementary Fig. 2A). In order to determine whether the corresponding sequences were deleted from intron
Fig. 3. Only LPAR6-mRNA from the c.756T > A (p.Tyr252*) mutant allele was expressed in the patient’s hair follicles. (A) Reverse transcription (RT)-PCR amplification of cDNAs derived from LPAR6 (top panel), RB1 (middle panel), and B2M (bottom panel) from the follicular RNA of the patient or a control individual. The B2M cDNA was amplified as a control. PCR without RT did not lead to any products, indicating that there was no genomic DNA contamination in the samples. +/− denotes reactions with or without RT. MMW, molecular weight markers; Pt, patient; C, control individual; (B) Direct sequencing of the LPAR6 cDNA amplified from the patient’s sample revealed only the c.756T > A (p.Tyr252*)-mutant LPAR6. (C) Schematic representation of the LPA6 protein. Transmembrane domains are indicated by yellow boxes. Position of the mutation p.Tyr252* is indicated by an arrow. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

![Graph](image1)

**Fig. 4. Improved expression and detection of LPA6 by using codon optimization and Ki16425 treatment.** (A) Cells transfected with the indicated N-terminally FLAG epitope-tagged LPA6 (native or codon-optimized sequence) or an empty vector (mock) were subject to flow cytometric analysis with an anti-FLAG antibody. FLAG-positive cells and mean fluorescent intensity are shown on the right (mean ± SD values of three culture replicates in a single assay). Data are representative of two independent experiments with similar results. (B, C) Cells transfected with AP-TGFs-encoding plasmid vector together with N-terminally FLAG epitope-tagged LPA6 (native or codon-optimized sequence) or an empty vector (mock) were harvested and seeded in a 96-well plate; the seeded cells were treated with the indicated concentration of LPA (B) or 1-alkyl-OMPT (C) for 1 h in the presence (+) or absence (−) of 10 μM Ki16425, a LPA1-3 inhibitor. Release of AP-TGFs into the conditioned media was quantified by ELISA. Spontaneous AP-TGFs release in a vehicle-treated condition was set at a baseline. Note that Ki16425 treatment suppressed LPA- and 1-alkyl-OMPT-induced responses in mock-transfected cells and that codon optimization enhanced LPA6 responses. Symbols and error bars indicate mean and SD values, respectively, of three culture replicates in a single assay. Data are representative of two independent experiments with similar results.
23 of RB1, we amplified this region by PCR and found that intron 23 was present in both RB1 alleles from the patient (Supplementary Fig. 2B).

3.3. Evidence for loss of transcription from the mutant LPAR6 allele with the insertion

To investigate how each mutation affected LPAR6 expression, we used total RNA samples from hairs plucked from the patient or a control individual to perform RT-PCR analysis. LPAR6 cDNA was amplified from each sample (Fig. 3A). However, direct sequencing of patient LPAR6 cDNA indicated that only LPAR6 cDNA from the c.756T > A (p.Tyr252*) was present; this finding indicated that the paternal mutant allele with the insertion in the LPAR6 promoter was not transcribed (Fig. 3B). In PCR for the RB1 cDNA, a clear single fragment was amplified from patient-derived or control-derived cDNA samples with similar efficiency. These findings indicated that the LPAR6 promoter insertion did not affect splicing of RB1 transcripts (Fig. 3A). Taken together, these results strongly indicated that only the p.Tyr252*-mutant LPA6 protein would be stably expressed in the patient’s HF.

3.4. Establishment of a sensitive assay system for LPA6

The c.756T > A (p.Tyr252*) mutation is located within the 6th transmembrane domain of the LPA6 protein (Fig. 3C). To investigate how the mutation affected the function, we tried to perform a series of in vitro analysis in cultured cells. We initially transfected an N-terminally FLAG-tagged WT LPA6 with native nucleotide sequences into HEK293FT cells and performed flow cytometry with an anti-FLAG antibody. However, to our surprise, the plasma membrane localization of the native LPA6 was undetectable (Fig. 4A). Furthermore, the native LPA6 poorly responded to LPA and 1-alkyl-OMPT (Fig. 4B and C). Through a series of improvement trials, we found a codon-optimized LPA6, which kept the amino acid sequences identical (Supplementary Fig. 1), efficiently localized to the plasma membranes (Fig. 4A) and the response to its agonists was also significantly enhanced (Fig. 4B and C). In addition, we found that pretreatment with an LPA1-3 antagonist Ki16425 almost completely suppressed the background response and improved the detection specificity of LPA6-dependent activation (Fig. 4B and C). Collectively, we have established a sensitive assay system for LPA6 and used it throughout this study.

3.5. The p.Tyr252*-mutant LPA6 failed to localize to plasma membranes and did not function as an LPA receptor

We transiently overexpressed either WT or p.Tyr252* (Y252X)-mutant LPA6 proteins and analyzed their plasma membrane localization. Cells that expressed WT LPA6 on cell surfaces were detected even when the amount of transfected expression construct was as low as 1 ng per well; the cell surface expression of WT LPA6 became more evident in a dose-dependent manner (Fig. 5). In contrast, expression of the Y252X-mutant LPA6 on cell surfaces was not evident in any sample, even when 100 ng per well of expression construct was used for transfection (Fig. 5). The results clearly showed that the Y252X-mutant LPA6 did not localize to plasma membranes.

We then investigated whether the p.Tyr252* (Y252X)-mutant LPA6 responded to LPA6 agonists. Both LPA and 1-alkyl-OMPT significantly activated the WT-LPA6 (Fig. 6). Notably, WT LPA6 responded to 1-alkyl-OMPT even when 0.1 ng of the expression construct was transfected per well (Fig. 6). However, the Y252X-mutant LPA6 did not show any response to either molecule; these findings indicated that the mutant protein did not function as an LPA receptor (Fig. 6).

3.6. The p.Tyr252*-mutant LPA6 disrupted the PA-PLA6/PLA6 axis

Finally, we used PA-PLA6α to test whether the p.Tyr252* (Y252X)-mutant LPA6 reacted to naturally occurring LPA species. As shown in previous studies [22,23,39], co-transfection of WT PA-PLA6α and WT LPA6 expression constructs markedly activated LPA6 signaling; however, this level of activation was not evident when WT LPA6 was co-expressed with a S154A-mutant PA-PLA6α; this recombinant mutant protein carried a critical amino acid substitution at a catalytic residue of PA-PLA6α protein (Fig. 7). By contrast, the Y252X-mutant LPA6 did not show any activity even when co-expressed with WT PA-PLA6α (Fig. 7). Taken together, these findings indicated that Y252X-mutant LPA6 failed to respond to endogenously produced LPA species.

![Graph](image-url)  
*Fig. 5. The p.Tyr252*-mutant LPA6 protein failed to localize at plasma membranes. Cells transfected with the indicated amount of N-terminally FLAG epitope-tagged LPA6 (wild-type (WT) or p.Tyr252* (Y252X)-mutant) or an empty vector (Mock) were subject to flow cytometric analysis with an anti-FLAG antibody. FLAG-positive cells and mean fluorescent intensity are shown on the right (mean ± SD values of three control replicates in a single assay). Data are representative of two independent experiments with similar results.
4. Discussion

In this study, we analyzed a Japanese family with ARWH/hypotrichosis and identified compound heterozygous LPAR6 mutations in the patient (Figs. 1 and 2). We initially searched for mutations in the LIPH gene because previously only LIPH mutations have been identified in Japanese patients with this disease [19,22,23,25,26]. Moreover, the clinical features of the affected individual analyzed in this study were indistinguishable from those of patients with LIPH mutations. To the best of our knowledge, we herein report the first Japanese case of ARWH/hypotrichosis caused by LPAR6 mutations, and our results further indicated a close relationship in function between the LIPH and LPAR6.

Of the two LPAR6 mutations identified in this patient, one was a large insertion mutation within the LPAR6 promoter region; this allele was paternally inherited (Fig. 2C–F). The insertion destroyed the structure of the promoter and resulted in loss of LPAR6 transcription from the mutant allele, as demonstrated by RT-PCR experiments (Fig. 3A and B). Interestingly, the inserted sequences were derived from intron 23 of the RB1 gene (Supplementary Fig. 2A). Our results indicated that a part of the sequences of intron 23 of the RB1 gene had accidentally been duplicated and inserted into the LPAR6 promoter, which is located in intron 17 of RB1.

Fig. 6. The p.Tyr252*-mutant LPA6 protein did not respond to LPA agonists. Cells transfected with AP-TGFα-encoding plasmid vector together with indicated amount of LPA6-encoding plasmid vector (wild-type [WT] or p.Tyr252* [Y252X]-mutant) or an empty plasmid vector (Mock) were harvested and seeded in a 96-well plate; the seeded cells were treated with the indicated concentration of 1-alkyl-OMPT or LPA for 1 h in the presence of 10 μM Ki16425. AP-TGFα release was determined as indicated in Fig. 4 legend and the method section. EC50 and Emax values were obtained by fitting data to a four-parameter logistic curve. Symbols and error bars indicate mean and SD values, respectively, of three culture replicates in a single assay. Data are representative of two independent experiments with similar results. Note that error bars are smaller than symbols in most data points. NA, not available.
The c.756T > A (p.Tyr252*) nonsense mutation identified in the maternal LPAR6 allele is predicted to generate a truncated LPA6 protein that lacks the C-terminus, including the 7th transmembrane domain (Figs. 2A and B and C). Based on the results of RT-PCR experiment, only the p.Tyr252* mutant LPA6 was expected to be expressed stably in the patient’s HF s. Previously, 24 distinct disease-causing mutations have been identified in the LPAR6 gene [8,9,27–37]. However, expression and functional analyses for these mutations have rarely been performed. Additionally, two premature termination codon mutations, p.C1n1555* and p.Lys125Asnfs*37, reportedly cause aberrant LPA6 localization within the cytoplasm [8]. Moreover, LPA reported does not activate the p.Lys125Asnfs*37-mutant LPA6 [8]. Similarly, the p.Tyr252* mutant LPA6 did not localize to plasma membranes (Fig. 5) and did not respond to any of the defined LPA6 agonists tested (Fig. 6). Finally, we clearly demonstrated that the p.Tyr252* mutant LPA6 disrupted the PA-PLAα/LPA/LPA6 axis, which caused ARWH/hypotrichosis in our patient (Fig. 7). We would like to emphasize that the activation of WT LPA6 (Figs. 4, 6 and 7) was detected much more sensitively than that shown in previous studies [8,13,14,22,23,39]. In this study, we have added two critical modifications into the in vitro assay system that we (A.I. and J.A.) have recently reported [13,14]: (1) we used codon-optimized LPA6 to significantly increase the efficiency of plasma membrane localization of recombinant LPA6 (Fig. 4A, Supplementary Fig. 1); (2) we treated the cells with an LPA1,- antagonist K16425 to inhibit the effect of endogenous LPA receptors expressed in HEK293FT cells [13] (Fig. 4B and C). As a result, we have finally established an assay system to sensitively detect LPA6 activation (Fig. 4). We believe that our assay system will become a useful tool to search for ideal agonists of LPA6 which can be used to produce a medicine for the treatment of ARWH/hypotrichosis in the future.

Our results provide important information regarding the molecular basis for ARWH/hypotrichosis in the Japanese population; they also further highlight the crucial roles of the PA-PLAα/LPA/LPA6 signaling in HF development and hair growth in humans.

Acknowledgements

We are grateful to Miho Morikawa for technical assistance with the TGFα shedding assay. This study was supported in part by a grant from Takeda Science Foundation, Japan (to Y.S.) and by “Research on Measures for Intractable Diseases” Project: matching fund subsidy (H26-077) from Ministry of Health, Labour, and Welfare, Japan. A.I. was funded by PRESTO (Precursory Research for Embryonic Science and Technology) from Japan Science and Technology Agency (JST) and a Grant-in-Aid for Scientific Research (C) (KAKENHI 26440043). J.A. was funded by CREST from JST and a Grant-in-Aid for Scientific Research on Innovative Areas from the Ministry of Education, Science, Sports and Culture of Japan (MEXT).

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.jdermsci.2015.03.006.

References


