TABLE 1. Clinical Characteristics of Familial Patients With *MAP2K2* Mutations

Reference	Patient no.	Age	Exon	*MAP2K2* mutation	Learning difficulty	Pulmonic stenosis	Cafe au lait spots
Classic CFC					Present	Present	Absent
Rauen et al. [2010]	V-2	7 months	3	p.Pro128Gln	N/A	Present	Absent
	IV-8	27 years			Present	Present	Present
	V-1	7 years			Present	Absent	Present
	III-6	42 years			Present	Absent	Absent
	II-2	78 years			Present	Absent	Absent
	III-3	58 years			[a]	Absent	Absent
	IV-5	18 years			Present	Absent	Absent
	IV-6	14 years			Present	Absent	Absent
Linden and Price [2011]	II-2	68 years	3	p.Gly132Asp	Absent	Present	Absent
	III-1	46 years			Present	Present	Absent
	III-3	40 years			Present	Present	Absent
The present report	Mother	31 years	6	p.Met223Val	Absent	Absent	Present
	Son	6 years			[b]	Absent	Present

CFC, cardio-facio-cutaneous syndrome.
[a]Tics.
[b]Patient was too young for a formal evaluation.

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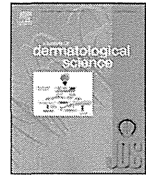

Identification of mutations in the prostaglandin transporter gene *SLCO2A1* and its phenotype–genotype correlation in Japanese patients with pachydermoperiostosis

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ABSTRACT

Background: Pachydermoperiostosis (PDP) is a rare genetic disorder characterized by 3 major symptoms: pachydermia including cutis verticis gyrata (CVG), periostosis, and finger clubbing. Recently, a homozygous mutation in the gene *HPGD*, which encodes 15-hydroxyprostaglandin dehydrogenase (15-PGDH), was found to be associated with PDP. However, mutations in *HPGD* have not been identified in Japanese PDP patients.
Objective: We aimed to identify a novel responsible gene for PDP using whole exome sequencing by next-generation DNA sequencer (NGS).
Methods: Five patients, including 2 patient-parent trios were enrolled in this study. Entire coding regions were sequenced by NGS to identify candidate mutations associated with PDP. The candidate mutations were subsequently sequenced using the Sanger method. To determine clinical characteristics, we analyzed histological samples, as well as serum and urinary prostaglandin E2 (PGE2) levels for each of the 5 PDP patients, and 1 additional patient with idiopathic CVG.
Results: From initial analyses of whole exome sequencing data, we identified mutations in the solute carrier organic anion transporter family, member 2A1 (*SLCO2A1*) gene, encoding prostaglandin transporter, in 3 of the PDP patients. Follow-up Sanger sequencing showed 5 different *SLCO2A1* mutations (c.940+1G>A, p.E427_P430del, p.G104*, p.T347I, p.Q556H) in 4 unrelated PDP patients. In addition, the splice-site mutation c.940+1G>A identified in 3 of 4 PDP patients was determined to be a

Abbreviations: CVG, cutis verticisgyrata; NGS, next-generation DNA sequencer; PDP, pachydermoperiostosis; SLCO2A1, solute carrier organic anion transporter family member 2A1; PGT, prostaglandin transporter; SNP, single nucleotide polymorphism.

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founder mutation in the Japanese population. Furthermore, it is likely that the combination of these *SLCO2A1* mutations in PDP patients is also associated with disease severity.
Conclusion: We found that *SLCO2A1* is a novel gene responsible for PDP. Although the *SLCO2A1* gene is only the second gene discovered to be associated with PDP, it is likely to be a major cause of PDP in the Japanese population.
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1. Introduction

Pachydermoperiostosis (PDP), also known as primary hypertrophic osteoarthropathy, is a rare autosomal recessive condition characterized by 3 major symptoms: cutis verticis gyrata (CVG), periostosis, and finger clubbing. In addition, several other symptoms, including sebaceous hyperplasia, hyperhidrosis, and arthropathy have also been reported [1,2]. The phenotypic spectrum of PDP is broad, and is generally categorized into 3 primary forms: the complete form, which involves all 3 major symptoms, including CVG; the incomplete form, which has all three symptoms but solely lacks CVG; and the "form fruste," characterized by the occurrence of pachydermia and minimal or absent skeletal changes [3].

To date, homozygous and compound heterozygous mutations in the *HPGD* gene, which encodes 15-hydroxyprostaglandin dehydrogenase (15-PGDH), have been identified as the main causative factor of PDP (MIM#259100) [4–10]. The primary function of 15-PGDH is an enzyme to catabolize for prostaglandin E2 (PGE2), prostaglandin F2 (PGF2), and prostaglandin B1 (PGB1). The identified *HPGD* mutation results in chronic elevation of PGE2 levels in serum, but it is unclear whether this elevation of PGE2 is associated with PDP phenotypes. Furthermore, several cases of PDP patients with congenital clubbed nails and *HPGD* mutations have also been reported [3–8]. We have also attempted to find *HPGD* mutations in Japanese PDP patients; however, no *HPGD* mutations have been identified so far, suggesting the existence of other causative gene(s) responsible for PDP in the Japanese population.

Recent advances in DNA sequencing techniques, such as the advent of next-generation sequencer (NGS), now allow for the analysis of all coding regions in exons (whole exome sequencing). In this study, we identified 5 different mutations in the solute carrier organic anion transporter family, member 2A1 (*SLCO2A1*) gene, which encodes prostaglandin transporter (PGT), in 4 unrelated PDP patients using whole exome sequencing and Sanger sequencing approaches. In addition, we assessed the potential impacts of the identified *SLCO2A1* mutations on disease severity and tested for associations between these variants and the clinical forms.

2. Patients and methods

2.1. Clinical report

PDP was diagnosed in the patients in our study, all of whom were of Japanese descent, on the basis of established clinical and radiological criteria [1]. All individuals participating in the study gave their written informed consent. This study was approved by the ethics committee of the National Center for Child Health and Development, and Keio University School of Medicine. *HPGD* mutation analyses had been performed previously [9], and no mutations were detected in any of the patients.

2.1.1. Patient1 (P1)

Clinical details for this patient have been reported in full elsewhere [11]. Briefly, at the age of 19, the patient was referred to evaluate his endocrinological status. He had a 6-year history of clubbing of fingers and toes. On physical examination, a coarse face, greasiness of facial skin (Fig. 1, P1), and hyperhidrosis were observed. Marked thickening of the scalp (CVG) was not evident. A skin biopsy specimen from the forehead skin showed thickening of the dermis. Interwoven collagen bundles, hypertrophic sebaceous glands, and increased density of sweat glands were subtle but evident in the dermis [11]. Elastic fibers and fibrosis were not observed only in the superficial dermis. Endocrinological examinations showed no notable findings. Radiological examination showed the presence of periostosis of the diaphysis of the radius, ulna, tibia, and fibula. On the basis of these observations, the patient was diagnosed with the incomplete type of PDP. At the age of 21, hydrarthros is developed in the knee joints. Swelling in knee joints was evident, but the patient did not complain of arthralgia or local joint heat. He was born with normal measurements following an uneventful pregnancy. None of the patient's immediate family members, including both parents and 2-year-old sister, had PDP or associated symptoms.

2.1.2. Patient 2 (P2)

This patient was 23 years old at the time of the study. At the age of 12, he noticed enlargement of fingers and toes, swelling of elbow and knee joints, as well as hyperhidrosis. At the age of 14, he presented with clubbing of fingers and toes, periostosis, and pachydermia. He was then diagnosed with PDP. At the age of 15, he was referred to one of the authors. Prominent swelling of the lower legs, paw-like fingers, and greasiness of the facial skin were observed. Radiological examination showed periostosis of the diaphysis of the radius and a cauliflower-like appearance of phalanx. Endocrinological examinations showed no notable findings. By the age of 23, the patient showed no clinical symptoms of CVG. He was diagnosed with the incomplete form of PDP. No skin biopsy specimen of this patient was available. The patient has no sibling, and his parents did not show any signs of the disease.

2.1.3. Patient 3 (P3)

The case of this patient has also been reported elsewhere [12]. At the time of the study, the patient was 41 years old. He first presented with thickening and furrowing of the scalp (CVG) and forehead (Fig. 1, P3), which the patient had noticed at the age of 17. His facial skin appeared greasy, and digital clubbing was apparent. Radiological examination showed periostosis of the diaphysis of the radius and ulna. Arthropathy was not evident. A skin biopsy specimen from the scalp and forehead (Supplementary Fig. 1) showed thickening of the dermis, which was filled with hypertrophic sebaceous glands and dense thickened collagen bundles. Abundant sweat glands and mucin deposition were also seen in the dermis. These findings met the diagnostic criteria of the complete form of PDP. His familial history was unavailable.

2.1.4. Patient 4 (P4)

The case of this patient has been reported elsewhere [13]. At the time of this study, the patient was 25 years old, and had a 7-year history of digital clubbing and acne on the scalp. He developed a peptic ulcer at the age of 14. Since the age of 22, the patient showed thickening and furrowing of the forehead skin and scalp. Physical examination showed digital clubbing, greasiness of facial skin, and

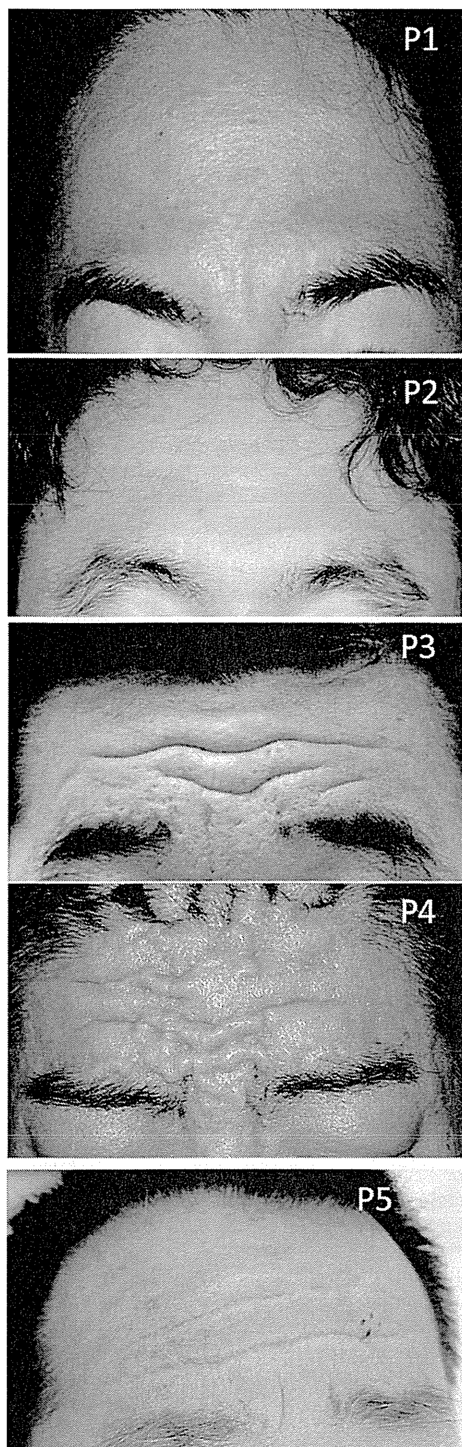

Fig. 1. Variation in forehead furrowing is associated with clinical forms of pachydermoperiostosis (PDP). Forehead furrows are apparent in the complete form of PDP, but less pronounced in the incomplete form. Greasiness of the skin was also evident in the complete form but not in the incomplete form of PDP.

hyperhidrosis of palms and soles. Pachydermia was prominent in the frontal, parietal, and occipital regions of the scalp as well as in the cheek and forehead skin (Fig. 1, P4). Endocrinological examinations showed no notable findings. Radiological examination showed periostosis of the diaphysis of the radius, ulna, tibia, and fibula. Arthropathy was not evident. A skin biopsy specimen (Supplementary Fig. 1) taken from the scalp and forehead showed thickening of the dermis. Thick and interwoven collagen bundles, sebaceous and sweat gland enlargement, and mucin deposits in the dermis were also prominent. These findings met the diagnostic criteria of the complete form of PDP. His familial history was noncontributory. The patient refused the examination after being informed.

2.1.5. Patient 5 (P5)

The case of this patient has been considered in another study [14]. Briefly, a 53-year-old man was referred to one of the authors. He had a 30-year history of digital clubbing and symmetric arthralgia of the knees. Physical examination showed transverse forehead furrows (Fig. 1, P5), but other skin manifestations, including seborrhea, acne, or hyperhidrosis were not evident. All laboratory tests, including thyroid function and serum levels of growth hormone, were within normal ranges, which ruled out thyroid acropathy and acromegaly. Magnetic resonance imaging of the brain showed CVG. Radiographic examination of the knee region showed periostosis with cortical thickening and ectopic ossification. Histological examination of the forehead skin showed acanthosis in the epidermis, sebaceous and sweat gland enlargement, and mucin deposits in the dermis. These findings met the diagnostic criteria of the complete form of PDP. His familial history was noncontributory.

2.1.6. Patient 6 (P6)

This patient was 52 years old when referred to one of the authors. He presented with furrows in the occipital region of the scalp that he had noticed since the age of 17. At the age of 30, he underwent plastic surgery to lift these furrows. He had swelling and pain of the joints with unsymmetrical manner. The patient had been treated for pain in his right acromioclavicular joint, which had been persisted for 3 years. Physical examination showed no digital clubbing or thickening of the forehead skin. No biopsy specimen was available. Folliculitis was evident in the occipital region of the scalp. He also showed hypertrophic gingiva in the lower jaw. Radiological examination showed no apparent periostosis of the diaphysis of the radius and ulna. The patient was diagnosed with idiopathic CVG. His familial history was noncontributory.

2.2. Measurement of prostaglandin E2 (PGE2) level in urine and serum

We examined serum and urinary levels of PGE2 using a commercial enzyme immunoassay kit (Cayman, Cayman Biochemical, Ann Arbor, MI, USA). Urinary and serum samples were stored in complete darkness at −30 °C until use.

2.3. Extraction of genomic DNA and total RNA

Genomic DNA was isolated from peripheral blood samples obtained from the patients and their parents using the QIAamp DNA Blood Maxi Kit (QIAGEN KK, Tokyo, Japan).

For reverse transcriptase (RT)-PCR analysis, total RNA was isolated from a skin sample of P3 using a commercial extraction kit (RNeasy Mini Kit; QIAGEN KK, Tokyo, Japan).

2.4. Whole exome sequencing

DNA fragments derived from exon regions were enriched using the SureSelect Human All Exon v4, according to the manufacturer's instructions (Agilent Technologies, Japan). The enriched DNA fragments were sequenced with the Illumina Genome Analyzer II according to the manufacturer's instructions, for 75 bp paired-end reads (Illumina, Japan). The raw image files were processed with

Illumina SCS2.8 software using the default parameters. Extracted DNA sequence reads were mapped to the human reference genome (hs37d5 assembly) using bwa [15]. Local DNA sequence alignment was processed by Picard to remove PCR duplicates. The Genome Analysis Toolkit (GATK) package was used to perform local realignment, map quality score recalibration, and make SNP/indel calls for each individual based on the following filter conditions: base quality greater than or equal to 20 and sequence depth greater than or equal to 4 [16]. Human transcript data in Ensembl database was used as gene model to evaluate mutations. For filtering of known SNPs, we used the Unified Genotyper module in GATK and data from public SNP databases, including dbSNP build 135, the 1000 Genomes Project pilot study, and our in-house Japanese SNPs dataset. Whole exome sequencing data of 100 Japanese controls, generated by the 1000 Genomes Project, were analyzed to calculate the frequency of the *SLCO2A1* mutation in the Japanese population (http://www.1000genomes.org/data).

2.5. Sanger sequencing analysis

We amplified and sequenced the entire coding region of *SLCO2A1* (NM_005630, 14 exons) to confirm mutations identified by whole exome sequencing (see Supplementary Table S1 for primer sequences and PCR conditions). The CodonCode Aligner (CodonCode Corporation, Dedham, MA, USA) was used for sequence data assembly and mutation confirmation.

2.6. RT-PCR and expression analysis

We analyzed the exon region surrounding an identified exon boundary mutation in P3 using RT-PCR. Total RNA was extracted from a skin biopsy specimen and reverse transcribed using an oligo-dT primer (Super Script®III First-Strand Synthesis System for RT-PCR; Invitrogen, Carlsbad, CA, USA). The PCR primer sets were designed to specifically amplify the transcribed region between exons 6 and 9 (see Supplementary Table S1 for primer sequence and PCR condition). Human Multiple Tissue cDNA (MTC) panels (Clontech, Palo Alto, CA, USA) were used for expression analysis of *SLCO2A1*.

2.7. Haplotype analysis

We analyzed 9 identified SNPs found by Sanger sequencing to determine haplotypes in the *SLCO2A1* region. For 2 pairs of patient-parent trios (P1 and P2), we determined each haplotype by comparing patient genotypes at the 9 SNPs to those of their parents. For P3, we treated as homozygous status because no heterogeneity was found in *SLCO2A1* region. For P5, 1 haplotype with mutation was deduced by comparison with haplotype I-ii. Finally we determined haplotypes of 16 alleles in 4 patients and their 2 parents.

3. Results

3.1. Variability in clinical features of PDP patients

According to Touraine's criteria for the use of clinical and radiological findings [1], 5 of the 6 patients were diagnosed with PDP (see Section 2 and summary in Table 1). All patients were male and had no familial history of PDP. All patients except P6 developed 1 of 3 major symptoms (also referred to as the "triad") before the age of 20, and subsequently suffered from all 3 symptoms of the triad. According to the classic clinical definition established by Touraine [3], the incomplete form of PDP consists of all triad symptoms, including pachydermia (on the forehead). Based on these criteria, only P1 and P2 were categorized as having the incomplete form; however, all PDP patients in this study (P1–P5) had pachydermia. Fig. 1 shows the clinical appearance of pachydermia in this study. A variety of skin folds on the forehead are seen. The typical appearance, characteristic of the complete form of pachydermia, is apparent in P3, P4, and P5, but it was not observed for P1. Furthermore, histological examination showed various degrees of sebaceous hyperplasia. This variability was associated with decreased density of elastic fibers and fibrosis surrounding sebaceous glands (Supplementary Fig. 1). Table 1 shows the results of histological examinations. It was clear that sebaceous hyperplasia was distinct in the complete form compared to the incomplete form; it should also be noted that minimal hyperplasia was evident from histological examination even in the incomplete form.

We further determined whether PGE2 levels in serum and urine were associated with the clinical forms, as increased PGE2 levels are likely to be a causative factor of PDP. The results clearly showed that high levels of serum PGE2 were detected in patients with the complete form of PDP (Table 1). Taken together, the results suggested that the differentiation of clinical forms of PDP is dependent on the presentation of clinical features, including pachydermia, its histology, as well as the PGE2 serum content.

3.2. Identification of SLCO2A1 *as a gene responsible for PDP*

In order to identify the responsible gene for PDP, we sequenced entire coding regions of 3 PDP patients (P1, P2, and P5) and 1 patient with only CVG (P6), which served as a disease control by whole exome sequencing. Approximately 100 million reads were quantified and mapped to the hs37d5 human reference genome DNA sequence, resulting in an average read depth of 70.0–96.5 for each individual whole exome sequencing (see Supplementary Table S2 for detail). We identified 36,392–41,957 variant sites compared to the reference sequence, of which 10,342–11,222 were splice site (SS) mutations or non-synonymous variants (NSVs). NSVs were further classified as nonsense (NS), start codon loss (SL), start codon gain (SG), frame shift (FS), and missense (MS) mutations. By filtering the data using public SNP databases, we finally identified 1–4 SS, 4–8 NS, 0–1 SL, 0–4 SG, 9–15 FS, and 124–157 MS mutations in the 4 patients (Table 2).

For subsequent mutation analysis, we focused on 5 genes in which NS, SL, SG, FS, and SS mutations were identified in at least 2 of 3 PDP patients. Detailed validation of mutations in these 5 genes showed that mutations in 3 of them were located in putative exons of minor transcript gene model in Ensembl database. The remaining genes (*SLCO2A1* and *ZNF98*) were selected as candidate genes for PDP. Using the IGV viewer [17], we analyzed entire coding region of these 2 candidate genes to assess DNA sequence quality and characterize all coding mutations. We found that all 3 PDP patients possessed compound heterozygous mutations in *SLCO2A1*: namely, P1 possessed a single SS mutation (c.940+1G>A) and a deletion of 4 amino acids (c.1279_1290del12, p.E427_P430del); P2 possessed an NS mutation (c.310G>A, p.G104*) and an MS mutation (c.1040C>T, p.T347I); and P5 possessed an SS mutation (c.940+1G>A) and an MS mutation (c.1668G>C, p.Q556H; Table 1). Although 2 PDP patients possessed a single heterozygous mutation (c.217delA) in *ZNF98*, this mutation was not found in other patients. We therefore identified *SLCO2A1* as a candidate gene responsible for PDP.

In order to confirm the mutations identified in *SLCO2A1*, we sequenced all 14 exons of the gene by Sanger sequencing in 4 PDP patients as well as in the parents of P1 and P2. We confirmed all of the *SLCO2A1* mutations identified by exome analysis (Fig. 2). In addition, we identified a homozygous SS mutation (c.940+1G>A) in P3 (Table 1). Each parent of P1 and P2 was found to be a carrier of 1 of the 2 mutations identified in P1 and P2.

Table 1
Summary of clinical phenotype, genotype and PGE2 contents.

Case	P1	P2	P3	P4	P5	P6
Current age (years)	24	25	45	37	53	52
Onset age (years)	13	12	17	10[§]	20	17
Clinical form	Incomplete	Incomplete	Complete	Complete	Complete	Unclassified[*]
HPGD	ND	ND	ND	NA	ND	ND
SLCO2A1 allele 1	c.940+1G>A	c.310G>A	c.940+1G>A	NA	c.940+1G>A	ND
	p.R288Gfs*7	p.G104*	p.R288Gfs*7	NA	p.R288Gfs*7	ND
SLCO2A1 allele 2	c.1279_1290del12	c.1040C>T	c.940+1G>A	NA	c.1668G>C	ND
	p.E427_P430del	p.T347I	p.R288Gfs*7	NA	p.Q556H	ND
Serum PGE2 (pg/ml)[**]	30	83	3880	NA	1762	647.4
Urinary PGE2 (pg/ml)	650	2940	68,160	NA	414.7	172.5
Triad						
Digital clubbing	+	+	+	+	+	–
Periostosis	+	+	+	+	+	–
Pachydermia	+	+	+	+	+	+
Cutis verticis gyrata	–	–	+	+	+	+
Skin						
Palmar and plantar hyperhidrosis	+	+	–	–	–	–
Acne	+	+	–	+	–	+
Seborhoea and eczema	+	+	+	+	–	–
Sebaceous hyperplasia with fiibrotic change surrounding space[@]	+/–[@@]	NA	+	++	+/–	NA
Skeletal						
History of bone fractures	–	–	–	+	–	–
Swelling of large joints	+	+	–	+	+	–
Painful joints on exercise	+	+	–	–	+[#]	+[#]
Hydrarthrosis	+	–	–	–	+[##]	–
Others						
Anemia	–	–	–	+	–	–
Peptic ulcers of the stomach and duodenum	–	–	–	+[§]	–	–
General fatigue	–	–	–	–	–	+
References	[11]	Present study	[12]	[13]	[14]	Present study

NA, not available; +, positive; –, negative or unknown; ND, (tested but) not detected.
[§] Treated with vagotomy at the age of 10.
[*] Having idiopathic cutis verticis gyrata (CVG).
[**] Normal range: 25–200 pg/mL.
[@] On histologic examination: ++, prominent; +, obvious; +/–, subtle.
[@@] Fibrotic change was observed only in superficial dermis.
[#] Active stage at current age.
[##] Arthroscopy revealed diffuse osteosclerosis and ectopic ossification.

Table 2
Variation summary statistics for whole exome sequencing of four PDP patients.

	Total variation	SS-NSVs	Known variation		Unknown variation					
			dbSNPs135	Other[a]	SS	NS	SL	SG	FS	MS
P1	38,746	10,484	9943	384	2	6	1	0	12	136
P2	36,392	10,342	9837	358	4	8	1	1	9	124
P5	40,539	11,037	10,492	389	2	6	1	2	10	135
P6	41,957	11,222	10,614	427	1	4	0	4	15	157

[a] 1K genome, JPN exome.

We also analyzed whole exome sequencing data of 100 Japanese individuals generated by the 1000 Genomes Project to ascertain the frequencies of these *SLCO2A1* mutations in the Japanese population. Although we found 6 known SNPs and 1 novel synonymous mutation in the *SLCO2A1* coding region, none of the *SLCO2A1* mutations identified in the PDP patients were found in the 200 Japanese control alleles (data not shown).

In summary, we identified *SLCO2A1* compound heterozygous mutations in 3 PDP patients, and *SLCO2A1* homozygous mutations in 1 PDP patient; no *SLCO2A1* mutation was found in P6, who shows only CVG in 3 major symptoms of PDP (Table 1).

To exclude the possibility that the c.217delA mutation in the *ZNF98* gene is a causative or a modifier mutation, we sequenced the coding region of *ZNF98* in 4 PDP patients as well as in the parents of P1 and P2. We found a single heterozygous c.217delA mutation in 2 of the PDP patients and one of the parents. However, c.217delA was not found in the other 2 PDP patients. Phenotypic comparisons indicated that no specific phenotype was associated with the c.217delA mutation. Therefore, we deduced that the c.217delA mutation of *ZNF98* does not have a modifier effect on PDP.

3.3. *Haplotype and a founder mutation analysis of* SLCO2A1

In this study, we characterized 4 alleles in 3 PDP patients with the c.940+1G>A SS mutation. In order to determine whether this mutation was a founder mutation, we analyzed SNPs in the *SLCO2A1* gene. On the basis of the analysis of 9 SNPs, we identified 6 haplotypes in the 46-kb region of the *SLCO2A1* gene (Table 3). Among the 4 alleles with the c.940+1G>A SS mutation, 8 of 9 SNPs are identical and only one SNP is different in one of 4 alleles. This SNP (rs10935090) is located in the most upstream region of *SLCO2A1* in exon1 among these 9 SNPs. In addition, we found that SNP (rs10935090) is localized different LD block compared with other 8 SNPs in Japanese HapMap data. These results suggest that

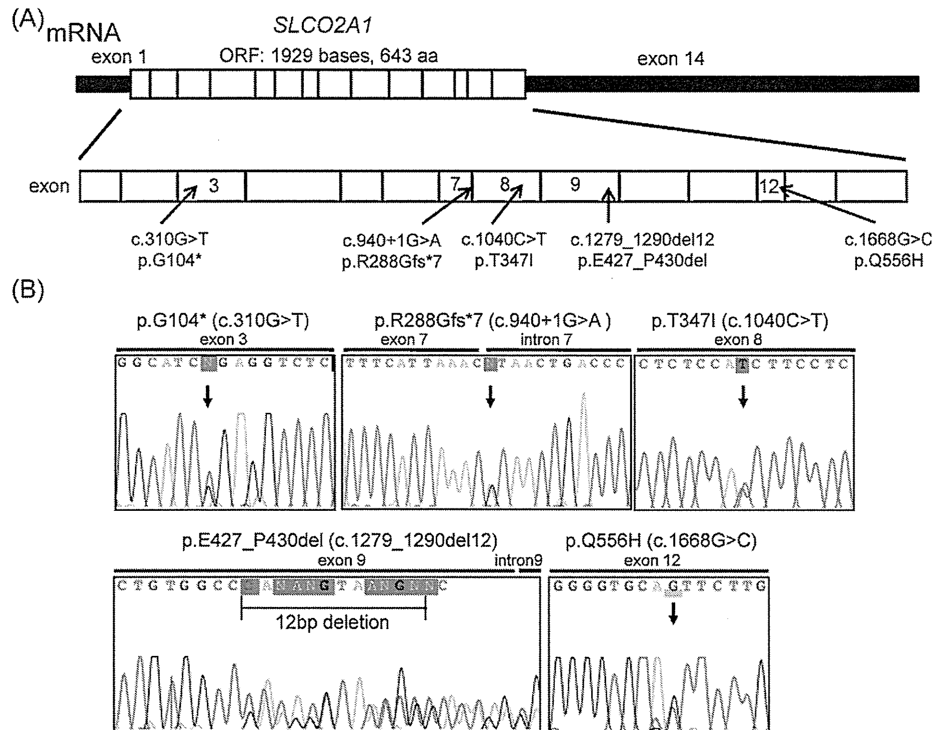

Fig. 2. Five mutations in *SLCO2A1* found by whole exome sequencing and confirmed using Sanger sequencing. (A) Five mutations in the *SLCO2A1* gene characterized by analysis of whole exome sequencing in this study. (B) *SLCO2A1* mutations confirmation by Sanger sequencing.

Table 3
Identified haplotypes and relation with mutation in SLCO2A1.

Haplotype	*SLCO2A1* mutation	rs10935090	rs4634113	rs6767522	rs6767412	rs55943046	rs34550074	rs2370512	rs1131597	rs1131598	
I-i	p.T347I	A	C	G	G	A	A	A	G	A	P2, P2fa
I-ii	p.R288Gfs*7	A	C	G	G	A	A	A	G	A	P1, P1fa, P3 (homo)
II	p.R288Gfs*7	G	C	G	G	A	A	A	G	A	P5
III	–	A	C	G	G	A	A	T	G	G	P2fa
IV-i	–	A	C	A	A	G	G	T	G	A	P1mo, P2mo,
IV-ii	p.G104*	A	C	A	A	G	G	T	G	A	P2. P2mo
V-i	–	G	C	A	A	G	G	T	A	A	P1fa
V-ii	p.Q556H	G	C	A	A	G	G	T	A	A	P5
VI	p.E427_P430del	G	C	G	G	A	A	T	G	G	P1, P1mo

fa, father; mo, mother.

the c.940+1G>A SS mutation in Japanese PDP patients is derived from a single founder mutant allele.

3.4. SLCO2A1 *transcript analysis for a c.940+1G>A splice site mutation*

In order to clarify the effect of the c.940+1G>A SS mutation of *SLCO2A1*, we analyzed *SLCO2A1* transcripts from a skin biopsy specimen obtained from P3 (homozygote of c.940+1G>A SS mutation) by RT-PCR. The c.940+1G>A SS mutation is located in the donor site of *SLCO2A1* intron 7, therefore, we designed an RT-PCR primer set to amplify a 396-bp fragment of cDNA between exons 6 and 9. However, the product generated from P3 cDNA was only ~300 bp (Fig. 3A). Sequencing of this shortened PCR product showed that the entire exon 7 (79 bp) was not included in the transcript (Fig. 3B–D). The loss of exon 7 resulted in a frameshift at amino acid position 288 and the introduction of a premature stop codon after 6 amino acid residues (p.R288Gfs*7). In contrast, exon 7 was consistently observed in PCR products generated from cDNA derived from 27 human tissues (Fig. 3A). Therefore, we concluded that the c.940+1G>A SS mutation of *SLCO2A1* resulted in the loss of PGT function.

4. Discussion

4.1. Mutation analysis

In this study, we performed whole exome sequencing of Japanese PDP patients and successfully identified *SLCO2A1* as a novel gene responsible for PDP. The *SLCO2A1* gene encodes a PGT protein with a total of 12 transmembrane domains. It is proposed that the PGT is a members of the "eicosanoid signaling" system, similar to the synaptic vesicle reuse system involved in neurotransmission [21]. In this system, PGT is thought to function the reuptake of prostaglandin into the cytoplasm, and then prostaglandin is digested by 15-PGDH. This hypothesis is compatible with the recent findings showing the genes encoding 15-PGDH and PGT are responsible for PDP. Therefore, dysfunction of the *SLCO2A1*

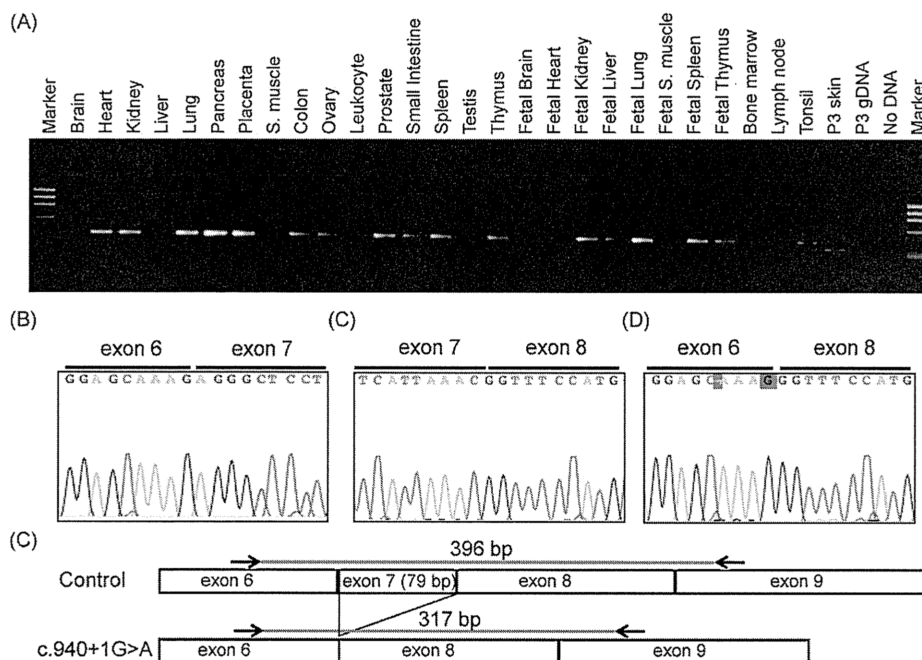

Fig. 3. Splice site mutation c.940+1G>A causes loss of exon 7 in *SLCO2A1* mRNA. (A) RT-PCR product amplified between exon 6 and exon 9 in *SLCO2A1*. Although *SLCO2A1* mRNA of the expected size is expressed in a wide variety of human tissues, only a smaller PCR product was obtained from the skin biopsy specimen taken from P3 who was homozygous for the c.940+1G>A SS mutation. (B–D) Sequence analysis of the PCR products generated between exon 6 and exon 9. The exon 7 region was detected in the exon boundaries of RT-PCR products from lung tissues (B, C). No exon 7 sequence was detected in the PCR product derived from skin mRNA of P3 (D). (E) Schematic representation of the exon 7 skipping. The *SLCO2A1* c.940+1G>A SS mutation located in the splice donor site of intron 7, which results in the loss of exon 7 and a truncation of PGT.

gene would cause the failure of PGE re-uptake, resulting in high PGE2 levels in the skin tissue, serum, and urine.

Of the 4 PDP patients screened, 1 carried a homozygous mutation, where as the other 3 carried compound heterozygous mutations in the *SLCO2A1* gene. All 4 PDP patients who possessed *SLCO2A1* mutations showed typical PDP phenotypes which are classified into the complete or incomplete forms. However, no significant mutations in the *SLCO2A1* gene were identified in the sole patient with CVG. Among the 8 alleles of *SLCO2A1* identified in this study, 4 (50%) included the same SS mutation (c.940+1G>A), which would be derived from a single founder allele. The other 4 *SLCO2A1* mutations were found in one of mutations in compound heterozygotes (summarized in Table 1). Analysis of *SLCO2A1* transcripts in P3, who was homozygous for the c.940+1G>A SS mutation, showed that the mutation resulted in the loss of exon 7 and a truncation of the PGT protein.

Among the 5 types of *SLCO2A1* mutations we identified, the NS mutation p. G104*and the c.940+1G>A SS mutation are considered to be severe mutations, as they result in truncations of the PGT protein. The p.Q556H mutation is located in the highly conserved 11th transmembrane domain adjacent to 1 of 3 critical amino acid residues (Glu78, Arg561, or Lys614) for PG transport activity [18] (Fig. 4). No information has been reported in previous investigations of PGT with regard to the potential functional impacts of the 2 mutations p.T347I and p.E427-P430del. Thr-347 is located in the extracellular region between the 7th and 8th transmembrane domains and is highly conserved in human, mouse, chicken, frog, and zebrafish (Fig. 4). The amino acid sequence containing the p.E427-P430del mutation (EVYP) is located in the extracellular region between the 9th and 10th transmembrane domains. The amino acid sequence (V/I)YP is conserved in human, mouse, chicken, and zebrafish, but not in frog (Fig. 4). Therefore, it is possible that the amino acid deletion mutation p.E427-P430del could have a less severe effect on PG transport activity.

Collectively, we deduced that the homozygous status of the c.940+1G>A SS mutation observed in P3, would have the most severe impact on PGT function. Compound heterozygotes with an c.940+1G>A SS mutation and a p.Q556H mutation, for example P5, would also be expected to have hindered PGT function, although to a lesser extent than c.940+1G>A homozygotes.

For *ZNF98*, we found a single heterozygous c.217delA mutation in 2 of the 4 PDP patients in one of their parents who did not have PDP. Phenotypic comparison indicated that no specific phenotype was associated with PDP patients who possess a single heterozygous c.217delA mutation. Therefore, we deduced that the c.217delA mutation of *ZNF98* does not have a modifier effect on PDP.

4.2. Genotype–phenotype correlation in PDP

SLCO2A1 mutations and clinical phenotypes of 6 patients are summarized in Table 1. Interestingly, P3, diagnosed with the complete form of PDP, was homozygous for the severe c.940+1G>A SS mutation, whereas P1, P2, and P5 were all found to be compound heterozygotes for *SLCO2A1* mutations represented both the incomplete form (P1, P2) and the complete form (P5) of PDP, carrying a severe mutation (either c.940+1G>A SS mutation or NS mutation) and another *SLCO2A1* mutation. The severity of pachydermia and associated histological changes was also correlated with *SLCO2A1* genotypes (Fig. 1 and Table 1). In addition, serum and urinary PGE2 levels in P3 were much higher than those observed for other PDP patients. Together, clinical data and genetic analyses showed that *SLCO2A1* genotypes in PDP patients were closely associated with serum PGE2 levels, suggesting that *SLCO2A1* mutations contribute to the severity of clinical phenotypes in PDP.

It was previously reported that transgenic mice with the K5 promoter – PTGS2 (also known as Cox2) transgene, exhibit high

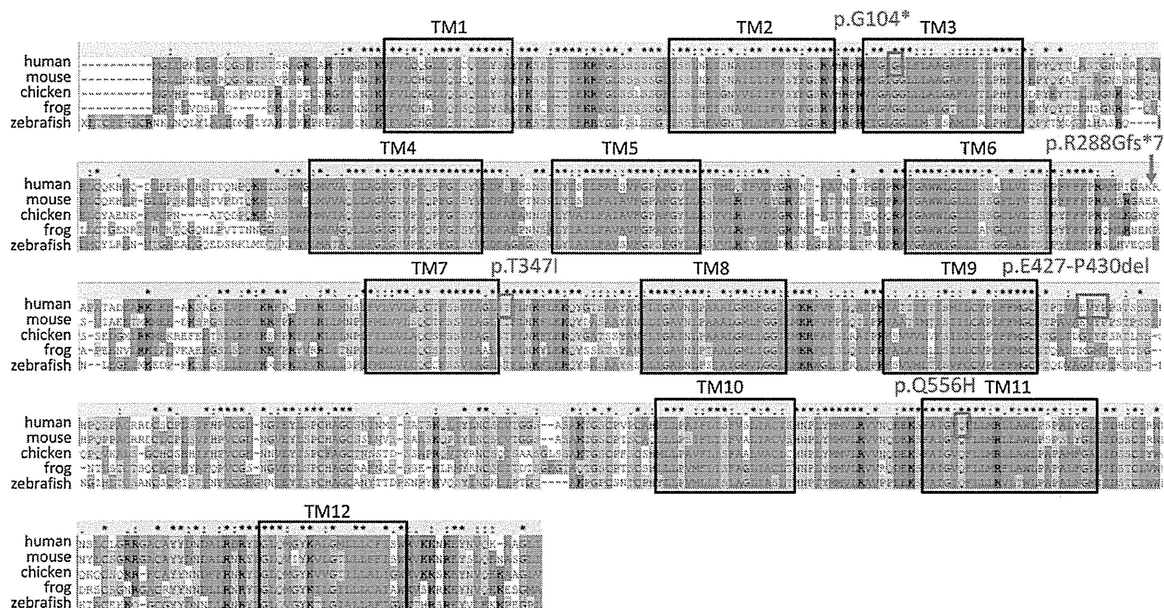

Fig. 4. Amino acid sequence alignments of PGT orthologs and mutations in PDP patients. Amino acid sequence alignments of PGT sequences of human (NP_005621) and 4 other vertebrate species (NP_201571, ENSGALG00000006471, ENSXETG00000011817, and ENSDARG00000061896) were performed using ClustalX (2.0). Twelve transmembrane regions were predicted using SOSUI ver1.11. The 5 *SLCO2A1* mutations found in this study are shown in red.

mRNA expression level of PGE2 in epidermis, epidermal hyperplasia, and sebaceous gland hyperplasia [22]. These hyperplasia phenotypes are quite similar to those of PDP patients. Furthermore, it was reported that PGE2 inhibits the proliferation of human gingival fibroblasts *in vitro* [23]. Therefore, high concentrations of PGE2 level in the skin tissue would cause epidermal, and sebaceous gland hyperplasia and dermis hypoplasia, and these cell proliferation differences between the dermis and epidermis would determine the magnitude of affected skin in PDP.

Although the serum PGE2s level of patients with the incomplete type PDP patients (P1 and P2) were within the normal range, we found mild sebaceous gland hyperplasia in P1 (Supplementary Fig. 1). These results suggested that the mild *SLCO2A1* mutation found in the patients with the incomplete form of PDP could alter the PGE2 level in affected skin, although serum PEG2 level would be within the normal range.

4.3. Founder effect of SLCO2A1 *mutation*

We found that 3 of the 4 patients possessed the c.940+1G>A SS mutation. All of these patients were unrelated and showed no consanguinity. In this study, we have shown that this mutation represents an ancient founder allele rather than a recurrent mutation (Table 3). These results indicated that c.940+1G>A SS mutation is one of major mutation in Japanese PDP patients and c.940+1G>A SS mutation should be analyzed first in all Japanese PDP patients before genetic screening at other *SLCO2A1* mutation. During manuscript preparation, 2 papers describing the identification of *SLCO2A1* mutations in PDP patients were published (MIM#614441) [19,20]. In one of these papers, Chinese PDP patients, who possess c.940+1G>A SS mutation, were reported. These results indicated that c.940+1G>A SS mutation would occur before divergence between Chinese and Japanese population, and currently spread in Asian area.

In this study, we identified 4 novel mutations of the *SLCO2A1* gene (p.G104*, p.T347I, p.E427-P430del, and p.Q556H) in 3 Japanese patients and also confirmed that parents of 2 of the patients were carriers of these mutations, implicating an autosomal recessive mode of inheritance. This information will be useful for genetic counseling. We also found evidence of genotype–phenotype correlations between *SLCO2A1* mutations and disease severity; however, further analyses are needed to clarify correlations among *SLCO2A1* genotypes, PGE2 level in skin, and the clinical forms. The patients described here with *SLCO2A1* compound heterozygous mutations, including those carrying the founder allele, might be useful for future investigations.

Note added in proof

Additional manuscript for isolation of *SLCO2A1* mutation in PDP patients were published [24] in revision.

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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.2012.07.008.

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