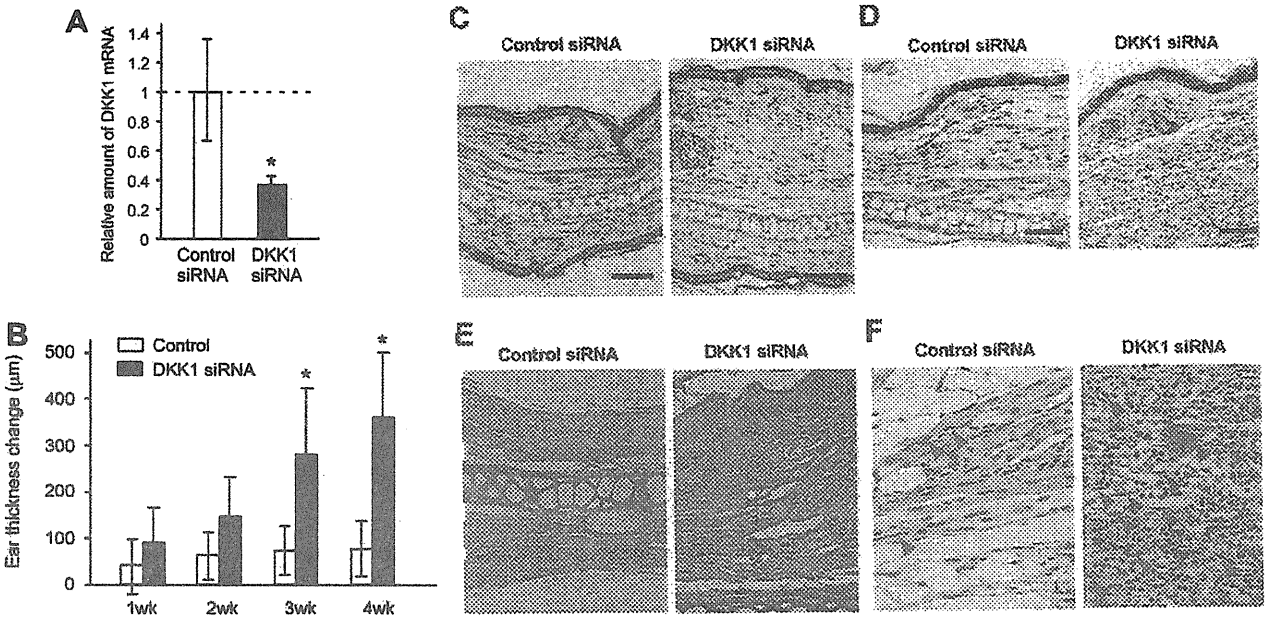

Figure 5. The effect of mouse *DKK1* siRNA on pachydermia. **A:** Mouse *DKK1* siRNA and control-scrambled siRNA (control siRNA) solutions were injected intradermally into the ears of mice four times every 7 days. The expression of *DKK1* mRNA in the skin 1 week after the last siRNA injection was evaluated by quantitative RT-PCR analysis. The mean value of *DKK1* mRNA relative to *GAPDH* mRNA treated with scrambled siRNA is regarded as one ($n = 5$). **B:** The ear thickness was measured every week before each injection and one week after the last injection. Columns show the mean ± *SD* ($n = 8$, each group) from two independent experiments. The student's *t*-test was performed between the indicated groups and $^*P < 0.05$. **C–D:** The skin from the ears one week after the last siRNA injection was fixed and stained with H&E (**C**). In addition, skin sections were stained with anti-β-catenin antibody by immunohistochemistry. Enhanced β-catenin expression is visible diffusely in the dermis of the skin treated with *DKK1* siRNA (**D**). Scale bars = 100 μm. **E:** The samples stained with Elastica van Gieson shows the thick collagen and elastic fibers in the dermis of mice treated with DKK1 siRNA. **F:** The samples are stained with proliferating cellular nuclear antigen (PCNA). **Red arrowheads** depict PCNA positive cells.

Hence, we further analyzed the sequence of *DKK1* and *TCF-4*. However, sequence analyses of the coding sequences of DKK1 and TCF-4, including exon-intron boundaries revealed no mutation (data not shown). In addition, the primers used in this study sequenced all exon-intron boundaries of *DKK1, TCF4*, and *HPGD*, but no mutation was found.

DKK1 *siRNA Enhances Ear Thickness in Mice*

Finally, we used mice to pursue direct evidence for DKK1 involvement in pachydermia. We injected a solution of mouse *DKK1* siRNA or control siRNA intradermally into the ears of mice four times every 7 days. Quantitative RT-PCR analysis revealed that this procedure successfully suppressed the expression of *DKK1* mRNA in the skin by about 60% (Figure 5A). The ear thickness was measured every week before each injection and 1 week after the last injection. The ear thickness was significantly augmented by the application of *DKK1* siRNA (Figure 5B). The histological findings showed that the dermis was thickened with increased fibroblasts (Figure 5, C–F). Consistent with these findings, enhanced β-catenin expression was observed diffusely in the dermis treated with *DKK1 si*RNA. (Figure 5D).

Discussion

We showed that Wnt/DKK1 plays a key role in the development of pachydermia in several aspects. Firstly, proliferation of fibroblasts from the PDP patients was promoted with a higher ratio in the cell cycle than compared with normal fibroblasts, and human recombinant DKK1 protein decreased their proliferation. Secondly, the expression levels of DKK1 mRNA in PDP fibroblasts and DKK1 protein in PDP skin were lower than those in healthy controls. Thirdly, β-catenin intensity in the skin from PDP was pronounced by immunohistochemistry. Finally, application of mouse *DKK1* siRNA increased the thickness of the skin in accordance with the elevated β-catenin levels. These results suggest that enhanced Wnt signaling is related to the development of pachydermia.

Pachydermia is one of the clinical manifestations of the complete form of PDP, which involves both skin and bone. For example, BMP, TGF-β, and Wnt families are the possible molecules responsible for the changes in both organs. There are several congenital diseases related to both organs, such as basal cell nevus syndrome, synovitis acne pustulosis hyperostosis ostitis syndrome, Klippel-Trenaunay-Weber syndrome, and Buschke-Ollendorff syndrome.[31–33] Bushchke-Ollendorff syndrome, in which osteopoikilosis is associated with connective tissue nevi, is particularly of note, since mutations in *LEMD3*, a gene implicated in BMP signaling, are candidates for its pathogenesis.[34] However, we could not detect a significant difference in mRNA expression for *BMP* or *TGF-β* families between PDP and control fibroblasts by DNA microarray analysis.

Recently, the incomplete form of PDP was attributed to elevated PGE_2 due to the mutation of *HPGD*. The skeletal phenotype of PDP, particularly clubbing and periostosis, can clearly be explained by elevated PGE_2, since it is well known that PGE_2 stimulates the activity of both os-

teoclasts and osteoblasts,[35] leading to bone deposition (periostosis) and resorption (acro-osteolysis), respectively. However, we could not detect a mutation in HPGD. In addition, the level of serum PGE_2 from one of our PDP cases (case 1) was within the normal range (data not shown). In fact, long-term therapeutic administration of exogenous PGE_2 for skin ulcers secondary to systemic sclerosis, arteriosclerosis obliterans, and Buerger diseases does not induce pachydermia, sebaceous hyperplasia, or velvet coloration of the skin as adverse effects. Moreover, the addition of PGE_2 into the fibroblast culture did not induce proliferation. Therefore, it remains unknown how the skin manifestations of PDP are induced.

Here we focused on Wnt signaling in the development of pachydermia. Fibroblasts from PDP skin and bone marrow-derived fibroblasts of PDP patients are known to grow faster than those of healthy donors.[26,36] The transfection of *DKK1* into cultured mouse fibroblasts, NIH3T3, blocked WNT2-induced cell growth and the WNT2-induced increase in uncomplexed β-catenin.[37] WNT3a induced motility and cytoskeletal rearrangement of NIH3T3 cells.[27] These previous reports suggest that enhanced Wnt/β-catenin signaling promotes fibroblast proliferation and cytoskeletal rearrangement. In fact, we found that the frequency of PDP fibroblasts in cycle was increased, and that actin bundle formation was more pronounced in PDP fibroblasts. Moreover, the addition of human recombinant *DKK1* consistently suppressed the fibroblast proliferation.

The source of DKK1 and how it works in the skin are issues that remain to be clarified. According to our immunohistochemical analysis, the major source of DKK1 in the skin seems to be fibroblasts, because the DKK1 expression in fibroblasts was low in PDP. Since DKK1 is a secreted antagonist and may affect bystander cells in the vicinity of fibroblasts, the dysregulated production of DKK1 possibly modulates the functions of not only fibroblasts but also other cells, such as keratinocytes and melanocytes. It was reported that high *DKK1* expression by dermal fibroblasts in the palms and soles inhibits the function of melanocytes via suppression of β-catenin and microphthalmia-associated transcription factor, and enhances keratinocyte proliferation.[16,17,38] Mice with an overexpression of *DKK1* in skin consistently lacked formation of appendages, such as hair follicles, and the mice had no skin pigmentation on the trunk.[18]

The role of DKK1 has been more extensively studied in bone than in the skin. DKK1 is known to inhibit osteoblast differentiation, and the overproduction of DKK1 was noted in osteolytic bone lesions of patients with multiple myeloma.[39] The elevated *DKK1* levels in bone marrow plasma and peripheral blood from the patients were correlated with the presence of focal bone lesions. Recombinant human *DKK1* inhibited the differentiation of osteoblast precursor cells *in vitro*.[40,41] These previous observations could explain the periostosis in PDP possibly secondary to decreased *DKK1* expression. Since fibroblasts and osteoblasts are derived from mesenchymal origin, they seem to share in common the mechanism of differentiation and proliferation. Although we did not address the relationship between DKK1 and the skeletal phenotype in PDP, it would be of interest to analyze the function of osteoblasts in PDP.

The next question is how Wnt signaling is enhanced. One possibility provided by our present study is the suppression of DKK1 expression in fibroblasts. The mechanism by which DKK1 is down-regulated in PDP remains to be elucidated. It can be hypothesized that there is a mutation in *DKK1* or molecules controlling *DKK1* expression, such as TCF-4. However, no mutation was detected in either exons of DKK1 or TCF-4 genes. Therefore, in the present study, we could not determine the genetic mechanism responsible for the complete form of PDP and/or pachydermia. Given the defect in PDP appears to altered expression of DKK1, it will be of interest in future studies to analyze the regulatory regions of DKK1, especially around the TCF binding sites, an issue which remains to be clarified.

On the other hand, the Wnt/β-catenin pathway is known to increase *DKK1* mRNA and protein, thus initiating a negative feedback loop.[42] It can be hypothesized that this negative feedback regulation might be dysregulated in PDP. Moreover, due to this negative feedback system, DKK1 can work as a tumor suppressor gene in some types of neoplasia.[42,43] Hypertrophic osteoarthritis is occasionally induced by a variety of thoracoabdominal, sometimes malignant, conditions. The relationship between decreased DKK1 expression and secondary hypertrophic osteoarthritis in association with malignancy may be an interesting issue to pursue.

It still remains unclear whether PDP in our cases could be attributed to the mutation in HPGD or not. Of note is that our cases were diagnosed as the complete form of PDP including pachydermia and adolescent onset, but that the cases with HPGD mutation had the incomplete form of PDP without pachydermia and with early onset (within the first year of their lives). The onset of the PDP is bimodal. The first peak is during the first year of the life and the second at the age of 15 years.[3,44] Therefore, the pathogenesis of PDP might be subdivided into at least two groups. However, further clinical studies in combination with HPGD mutation analysis will be required to clarify this.

In PDP, clinical cutaneous manifestations include pachydermia, seborrhea, and velvet colored skin. At present, we could not show direct evidence that all of the phenotypes of PDP were induced by enhanced Wnt signaling secondary to the suppressed expression of DKK1. In addition, the number of cases in our study was limited. However, our findings, together with those of previous studies suggest that the Wnt signaling pathway was promoted in accordance with decreased DKK1 expression, leading to increased fibroblast proliferation, enhanced pigmentation of the skin, and adnexal hyperplasia.

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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 characterics, 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 (PGT), in 3 of the PDP patients. Follow-up Sanger sequencing showed 5 different *SLCO2A1* mutations (c.940+1G>A, p.E427_P430del, p.G104Ter, 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 founder mutation in the Japanese population.

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.

Keywords: PDP, SLCO2A1, Mutation analysis, Prostaglandin transporter

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

Running title: *SLCO2A1* mutations in Japanese pachydermoperiostosis

1. Introduction

Pachydermoperiostosis (PDP), also known as primary hypertrophic osteoarthropathy, is a rare genetic disorder 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 involves 2 of 3 major symptoms, excluding 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 [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.

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 [9] had been performed previously, and no mutations were detected in any of the patients.

Patient 1 (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, hydrarthrosis 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.

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.

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.2) 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.

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 hyperhidrosis of palms and soles. Pachyderma 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.2) 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.

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.

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 mRNA

Genomic DNA was isolated from peripheral blood samples obtained from the patients and their parents in 2 families (P1 and P2) 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 target enrichment system, 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.

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 CodonCodeAligner (CodonCode Corperation, 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 mRNA was extracted from a skin biopsy specimen and reverse transcribed using an oligo-dT primer (SuperScript® 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 "Patients and methods" 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 to 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. 2). 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), frameshift (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).