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 visually 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.Glu427_Pro430del); P2 possessed an NS mutation (c.310G>A, p.G104Ter) and an MS mutation (c.1040C>T, p.T347I); and P5 possessed an SS mutation (c.940+11G>A) and an MS mutation (c.1668G>C, p.Q556H; Table 1). In addition, 2 PDP patients possessed a single mutation in *ZNF98*. 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. 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).

*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+11G>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 exon 1 among these 9 SNPs. In addition, we found that rs10935090 is localized different LD block compared with other 8 SNPs in Japanese HapMap data. These results suggest that the c.940+11G>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 is resulted in a frameshift after amino acid position 287 and the introduction of a premature stop codon. 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 identified *SLCO2A1*, which encodes the PGT protein, as a novel gene responsible for PDP. Of the 4 PDP patients screened, 1 carried a homozygous mutation, whereas 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.G104Ter 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]

(Supplementary Fig. 1). 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 [18]. 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 (Supplementary Fig. 1). 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 xenopus (Supplementary Fig. 1). 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.

*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, 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.

*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 [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 be occurred 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.G104Ter, 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, 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.

## CONFLICT OF INTEREST

The authors state no conflict of interest.

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**Figure legends**

**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.

**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.

**Fig.3 Splice site mutation c.940+1G>A causes loss of exon 7 in *SLCO2A1* mRNA.**

(a) RT-PCR product amplified between exons 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 exons 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 deletion.

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.

Supplementary Figure 1

Amino acid sequence alignment analyzed by ClustalX (2.0), including PGT sequences of human and 4 other vertebrate species. Transmembrane regions were predicted using SOSUI ver1.11. The 5 *SLCO2A1* mutations found in this study are shown in red.

Supplementary Figure 2

Hematoxylin and eosin staining of skin biopsy specimens show that sebaceous hyperplasia is prominent in P4 (*right lower panel*), evident in P3 (*right upper panel*), and subtle in P1 (*left panel*). P1 was diagnosed with the incomplete form, and P3 and P4 were diagnosed with the complete form of PDP (Table 1). Notably, the magnitude of sebaceous hyperplasia is associated with the disappearance of elastic fibers and the degree of fibrotic change in the interstitial spaces.