Clin Genet 2008: 73: 373–379
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CLINICAL GENETICS
doi: 10.1111/j.1399-0004.2008.00975.x

Short Report

Three novel mutations in the *PORCN* gene underlying focal dermal hypoplasia

Leoyklang P, Suphapeetiporn K, Wananukul S, Shotelersuk V. Three novel mutations in the *PORCN* gene underlying focal dermal hypoplasia. Clin Genet 2008: 73: 373–379. © Blackwell Munksgaard, 2008

Focal dermal hypoplasia (FDH) is an X-linked dominant disorder characterized by patchy dermal hypoplasia with digital, ocular and dental abnormalities. Very recently, mutations in the *PORCN* gene were demonstrated to cause FDH. Here, we described three unrelated Thai girls who were sporadic cases of FDH. One of them had unilateral athelia, which has never been described in FDH. Mutation analysis by polymerase chain reaction sequencing the entire coding regions of *PORCN* successfully revealed three potentially pathogenic mutations, c.373+1G>A, c.737_738insA and c.1094G>A (p.R365Q). One was found in each of three patients. In addition, another sequence variant c.682C>T (p.R228C) with an inconclusive role was found in one patient and her unaffected mother. The two missense mutations were not detected in at least 100 ethnic-matched control chromosomes, and all four mutations had never been previously described. X chromosome inactivation studies showed random patterns in all of them. This study demonstrates that *PORCN* is the gene responsible for FDH across different populations and extends the total number of confirmed mutations to 26.

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Key words: focal dermal hypoplasia – Goltz syndrome – mutation – *PORCN* – X inactivation

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Received 23 October 2007, revised and accepted for publication 22 January 2008

Focal dermal hypoplasia (FDH) also known as Goltz syndrome (OMIM #305600) is an X-linked dominant disorder characterized by patchy dermal hypoplasia with herniation of fat through the dermal defects. Other typical findings include papillomas, sparse and brittle hair, and dystrophic nails. Digital anomalies consisting of syndactyly, polydactyly, camptodactyly or ectrodactyly and ocular anomalies such as colobomas, microphthalmia or anophthalmia are often found. Variable features include short stature, cleft lip and palate, hypodontia, and mental retardation (1). Very recently, mutations in the *PORCN* gene were demonstrated to cause FDH. So far, 23 disease-causing mutations in *PORCN* associated with FDH have been identified (2, 3). Here, we describe three Thai girls who are sporadic cases of FDH with novel mutations in the *PORCN* gene. In addition, balanced X inactivation pattern was detected in all of them.

Materials and methods

Clinical subjects

Informed consent was obtained for publication of the identifiable photographs. Patient 1 was a 6-year-old Thai girl born at term to a 35-year-old gravida 1, para 0 mother and a 35-year-old unrelated father. The birth weight was 2490 g and head circumference was 30 cm. She presented to us with streaks of markedly thinned dermis with fat herniation, bilateral microphthalmia and aniridia, incomplete cleft lip, absence of right nipple, ectrodactyly with four digits on her right hand and right foot, syndactyly on all extremities, and dysplastic nails (Fig. 1, left panel). A diagnosis of FDH was given. Chest radiography showed deformities of her fourth to sixth right anterior ribs. Echocardiography and ultrasonography of her kidneys showed no abnormalities. Hearing tests by brain stem auditory evoked potentials

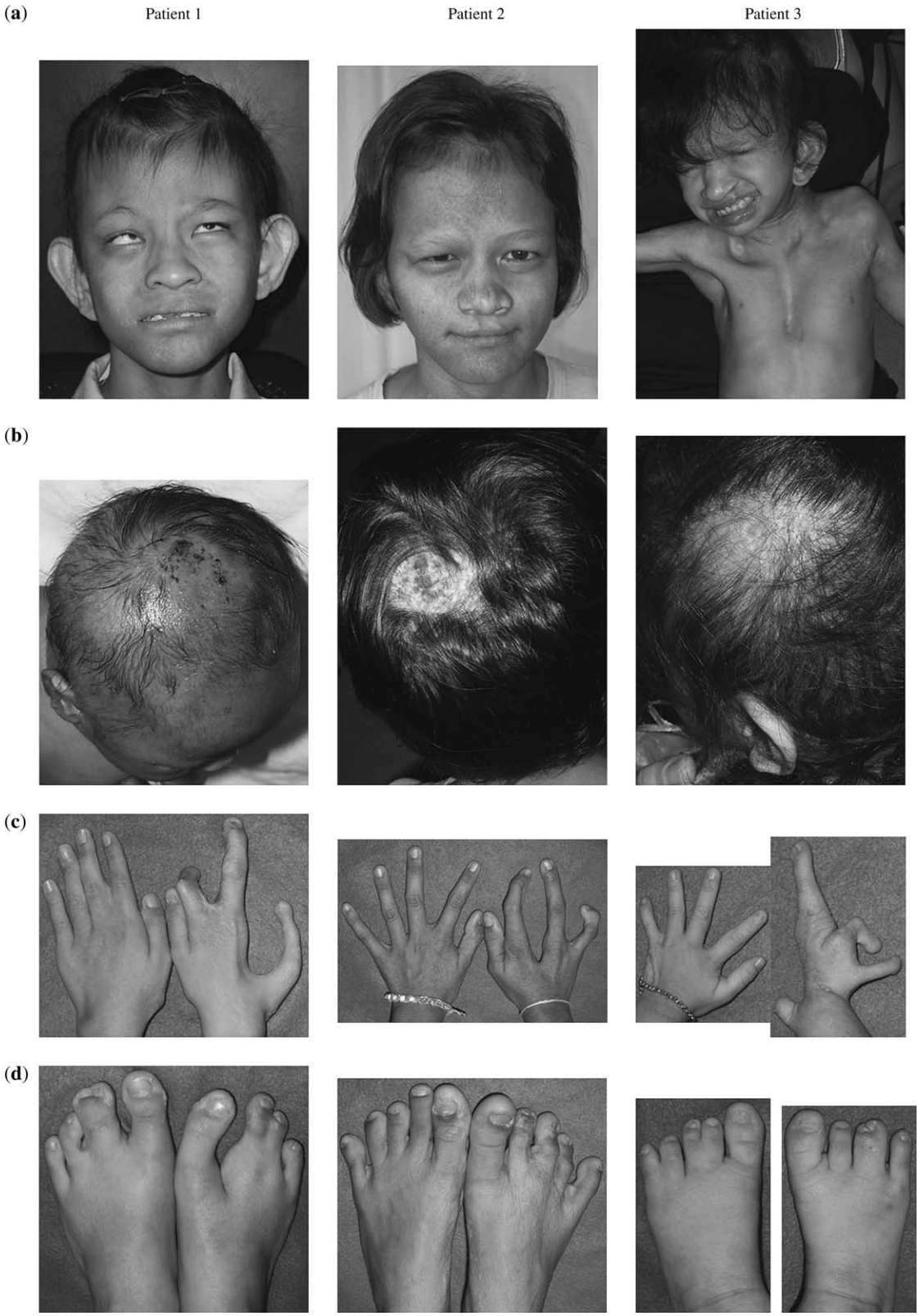

Fig. 1. Clinical features of the three Thai patients with focal dermal hypoplasia. Patient 1 at 6 years of age except that of the scalp defect which was taken at 3 days old, left panel; patient 2 at 14 years of age, middle panel; patient 3 at 3 years of age, right panel. (**a**) Face of the three patients and anterior chest wall of patient 3 showing split sternum. (**b**) Scalp defects. (**c**) Hands. (**d**) Feet.

were normal. Chromosomal analysis revealed 46,XX. She had gastroesophageal reflux and recurrent aspiration pneumonia, requiring a gastrostomy tube feeding during the age of 6 months and 4 years. Developmental assessment by the Gesell Developmental Schedule showed a mental age of 20 weeks at a chronological age of 40 weeks. However, the subsequent IQ test at the chronological age of 6 years showed that her IQ was in a normal range. On her last visit at age 6 years, her height was 114 cm (mean), weight 14.2 kg [−2 standard deviation (SD)], and head circumference 44.5 cm (−4 SD).

Patient 2 was a Thai girl born at term with a birth weight of 2200 g to a 24-year-old gravida 2, para 1 mother and a 25-year-old father. The parents were second cousins. The pregnancy and labor were unremarkable. She was found to have multiple anomalies since birth. However, no diagnosis was made at that time. She has been generally healthy. She first presented to us at the age of 14 years. She was small with a height of 142 cm (−2.5 SD), weight of 28 kg (−2.5 SD), and head circumference of 48 cm (−4 SD). She had generalized linear and patchy discolored lesions on her dry skin. She also had dermal aplasia on vertex measuring 4 × 5 cm, sparse eyelashes and eyebrows, midface hypoplasia, repaired incomplete left-sided cleft lip, hypodontia, ectrodactyly of her right hand, and dysplastic toenails (Fig. 1, middle panel). Ophthalmic evaluation revealed right microphthalmia and left choroidal colobomas. Chest radiograph and urine analysis were normal. She was in grade 8 in a regular class with satisfactory test results.

Patient 3 was a Thai girl born at term with a birth weight of 2300 g to a 35-year-old gravida 1, para 0 mother and a 31-year-old unrelated father. The pregnancy was unremarkable. She was born by cesarean section because of breech presentation. At birth, she had multiple anomalies and had been diagnosed with Goltz syndrome. She first sat at 8 months old. At age 3 years, she was able to walk and say her first word. Physical examination when she was first referred to us at 3 years and 8 months old revealed her weight of 8 kg (−3 SD) with bilateral anophthalmos, protruding ears, small right ear lobule, surgically repaired left complete cleft lip and palate, hypodontia, notched incisors, split sternum, ectrodactyly of the right hand, absent nail of the left second finger, syndactyly between the third and the fourth toes of the right foot and syndactyly between the second and the third toes as well as between the fourth and the fifth toes of the left foot, mild scoliosis, and linear and patchy dermal hypoplasia on the chest, back, and extremities (Fig. 1, right panel).

There were no histories of other family members being affected with the syndrome, frequent miscarriages, or male stillbirths in all three families.

Mutation analysis

Written informed consent was obtained from all patients and their parents included in the study. Genomic DNA was extracted from peripheral leukocytes according to standard protocols. Intronic primers as previously described were used to amplify fragments encompassing all the coding exons 2–15 of the *PORCN* gene (2). Polymerase chain reactions (PCRs) were carried out in a 20 μl volume containing 100 ng genomic DNA, 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 mM of each primer, and 0.5 U Taq polymerase. The PCR products were treated with ExoSAP-IT (USP Corporation, Cleveland, OH) according to the manufacturer's recommendations and sent for direct sequencing at Macrogen Inc. (Seoul, Korea). Analyses were performed by SEQUENCHER 4.2 (Gene Codes Corporation, Ann Arbor, MI). When the result indicated a possible new variant, the sample was resequenced.

Restriction enzyme analysis

Restriction enzyme digestion of the PCR products was used to screen for the non-synonymous coding variant, c.682C>T (p.R228C), in 97 normal Thai controls (65 females and 32 males), totaling 162 X chromosomes. Exons 6 and 7 of *PORCN* were PCR amplified using a mutagenic forward primer to incorporate a *Hha*I recognition site, 5′-CTTCCAGTACTTCATCCCCCT-CAACGGTGACCGCCTCCTG-3′ and a reverse primer, 5′-GTTGACGGTCAGACACACTT-3′. The PCR condition was 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C for 35 cycles. The PCR products were digested with *Hha*I according to the manufacturer's specifications (New England Biolabs, Ipswich, MA) and electrophoresed on a 3% agarose gel stained with ethidium bromide.

The non-synonymous coding variant, c.1094G>A (p.R365Q), was studied by restriction enzyme digestion of the PCR products of 50 Thai unaffected female controls. Exon 13 of *PORCN* was PCR amplified using the PCR condition of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C for 35 cycles. The PCR products were subsequently digested with *Bpm*I according to the manufacturer's

specifications (New England Biolabs) and electrophoresed on a 2% agarose gel stained with ethidium bromide.

Phase determination of c.682C>T and c.737_738insA

Using genomic DNA of patient 2 as template, exons 6–9 of the *PORCN* gene were PCR amplified using the forward primer for exon 6 and the reverse primer for exon 9. The predicted size of the PCR product was 1954 bp. The PCR products were then purified using a QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA) and subsequently subcloned into the cloning vector, pGEM-T Easy vector (Promega, Madison, WI). The resulting plasmids were transformed into *Escherichia coli* DH5-α competent cells (Life Technologies, Gaithersburg, MD) and purified using a QIAprep Spin Miniprep Kit (QIAGEN). The recombinant plasmids were investigated by PCR-restriction enzyme digestion and sequencing.

X chromosome inactivation analysis

The X chromosome inactivation study of peripheral blood leukocytes (PBL)-derived DNA was performed by PCR analysis of a polymorphic CAG repeat in the first exon of the *androgen receptor* (*AR*) gene. Methylation of *Hpa*II sites in close proximity to this repeat correlates with X chromosome inactivation (4). About 500 ng of genomic DNA was digested with 10 U of the methylation-sensitive enzyme *Hpa*II and incubated at 37°C overnight. After digestion, the reactions were terminated by incubating the mixture at 95°C for 10 min. The first exon of the *AR* gene was PCR amplified using a forward primer, 5′-FAM-CGCGAAGTGATCCAGAA CCC-3′ and a reverse primer, 5′-GTTGCTGTT CCTCATCCAGG-3′. PCR products from undigested and digested DNA were sent for size determination at Macrogen Inc.. Analyses were performed by GENEMAPPER version 3.7 (Applied Biosystems, Foster City, CA).

Protein sequence comparisons

PORCN orthologues were first identified through a BLAST search of the non-redundant database using *Homo sapiens* PORCN, accession NP_982301, as the reference sequence. All known and complete PORCN sequences were included from the vertebrate lineage. These files in FASTA format were then analyzed by CLUSTALX 1.81 program. The human PORCN was aligned with chimpanzee (*Pan troglodytes*; XP_521046), dog (*Canis lupus familiaris*; XP_851011), house mouse (*Mus musculus*; NP_076127), chicken (*Gallus gallus*; ABH12079) and African frog (*Xenopus tropicalis*; NP_989365). The program classified amino acids by the variation in polarity, assessing both amino acid class conservation and evolutionary conservation at any given site.

Results

PCR-sequencing analysis of the entire coding sequences of *PORCN* revealed that patient 1 was heterozygous for a c.373+1G>A mutation at the exon–intron splice junction GT of intron 4 (IVS4+1G>A; Fig. 2, left panel). This variant was predicted to result either in aberrant splicing involving exon 4 resulting in a mutant PORCN

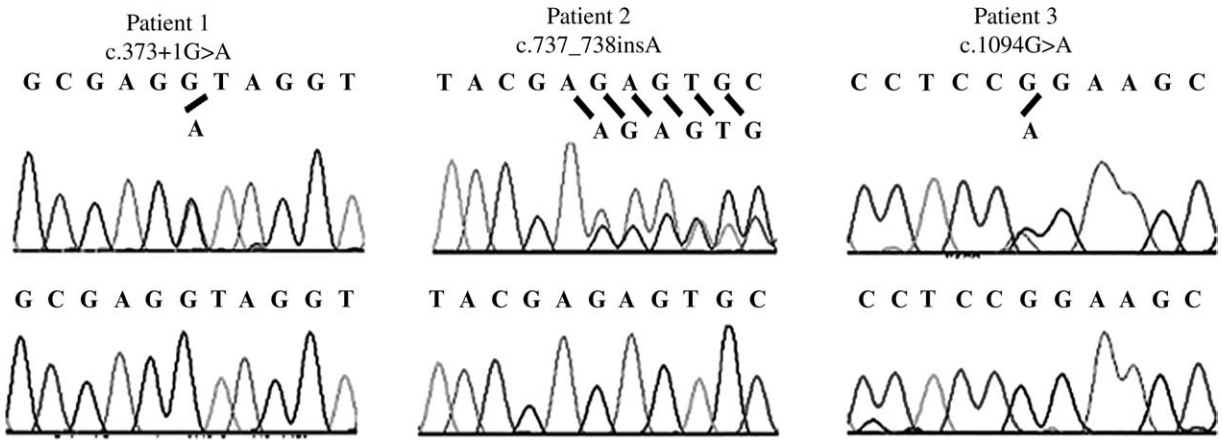

Fig. 2. Mutation analysis: the left, middle and right panels relate to c.373+1G>A, c.737_738insA and c.1094G>A (p.R365Q) mutations, respectively. Upper and lower panels are electropherograms of patients and controls, respectively.

protein or nonsense-mediated decay, in which the entire transcript was removed to avoid producing a truncated protein. The mutation was not found in her parents. No other DNA changes in the coding region of *PORCN* were found.

Patient 2 was found to be a heterozygote for two DNA changes. The first is a C>T transition at nucleotide position 682 (c.682C>T) in exon 6 (Fig. 3a). The mutation was confirmed by digestion of the PCR products with the restriction enzyme *Hha*I (Fig. 3c). The patient's clinically unaffected mother was also heterozygous for the c.682C>T (data not shown). Her father was not available for the study. The c.682C>T transition was expected to result in conversion of an arginine at codon 228 to a cysteine (p.R228C). The arginine is evolutionarily conserved in chimpanzee, dog, house mouse, and African frog but not in chicken, which has a proline at the codon. The c.682C>T was not detected in 162 ethnic-matched control X chromosomes.

Patient 2 was also heterozygous for an insertion of adenine between nucleotide positions 737 and 738 (c.737_738insA) in exon 9 of *PORCN* (Fig. 2, middle panel). The mutation is expected to result in alterations starting from codon 247 and a frameshift with a stop codon at codon 315 (p.S247EfsX315). The c.737_738insA was not found in her mother.

By cloning PCR products using DNA of patient 2 as template into a vector, we were able to determine that the c.682C>T (p.R228C) and the c.737_738insA were on the same allele.

Patient 3 was heterozygous for a c.1094G>A transition in exon 13 (Fig. 2, right panel). The mutation was confirmed by digestion of the PCR products with the restriction enzyme *Bpm*I (data not shown). Her parents had only the wild-type G (data not shown). The c.1094G>A mutation was expected to result in conversion of an arginine at codon 365 to a glutamine (p.R365Q). The arginine is a polar and positively charged amino acid that is different from glutamine, an uncharged polar amino acid. The arginine at codon 365 is evolutionarily conserved in chimpanzee, dog, house mouse, chicken, and African frog. The 1094G>A was not present in 100 ethnic-matched control X chromosomes. No other DNA changes in the coding region of *PORCN* were found in the patient.

X chromosome inactivation analysis revealed that X chromosome inactivation ratio in blood was 61:39, 57:43 and 44:56 in patients 1, 2 and 3, respectively (Fig. 4). The X inactivation ratio of the mothers of patients 1, 2, and 3 were 59/41, 82/18 and 44/56, respectively (Fig. 4).

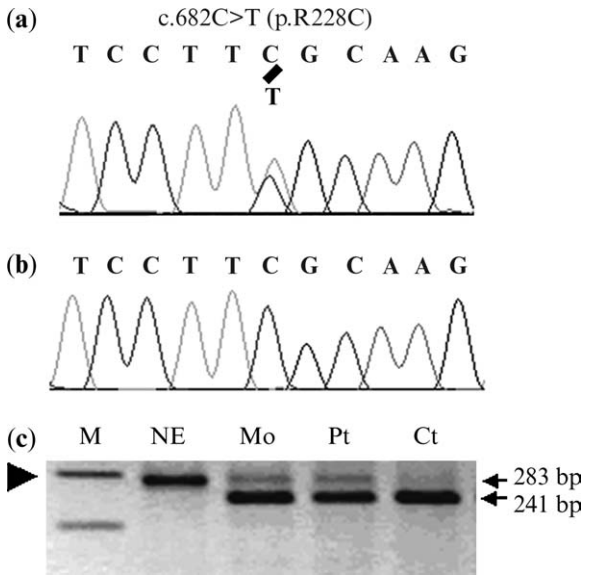

Fig. 3. The c.682C>T mutation (p.R228C) with inconclusive role found in patient 2 and her unaffected mother. The sense sequence electropherogram showing (**a**) the c.682C>T mutation in the patient and (**b**) normal CC in a control. (**c**) Restriction enzyme digestion analysis. Ct, unaffected control; M, 100-bp marker; Mo, mother of patient 2; NE, polymerase chain reaction (PCR) products of a control without addition of the restriction enzyme; Pt, patient 2. The 300-bp band is indicated by an arrowhead. Restriction enzyme analysis of the PCR products showing the mutant allele lacking the cleavage site for the restriction endonuclease *Hha*I resulting in an uncut 283-bp band. The wild-type alleles are digested resulting in 241- and 42-bp bands (the 42-bp band is not visualized).

Discussion

We described three unrelated Thai girls who were sporadic cases of FDH with novel mutations in the *PORCN* gene. The fact that our patients are all girls is consistent with the previous observation that 90% of patients with Goltz syndrome are female (2, 3, 5). All our three patients had streaks of markedly thinned dermis, the required features of FDH. Digital deformities, ocular anomalies, microcephaly, cleft lip with or without cleft palate, hypodontia, short stature and rib anomalies present in at least one of them have been previously observed as part of FDH (1, 6). Although breast hypoplasia was previously seen in one patient with molecular-confirmed FDH (2), unilateral athelia found in patient 1 has, to our knowledge, never been reported in the literature.

A heterozygous mutation for a c.373+1G>A in the 5′ end of intron 4 of *PORCN* (IVS4+1G>A) was identified in patient 1. The mutation was not found in her parents. This mutation has

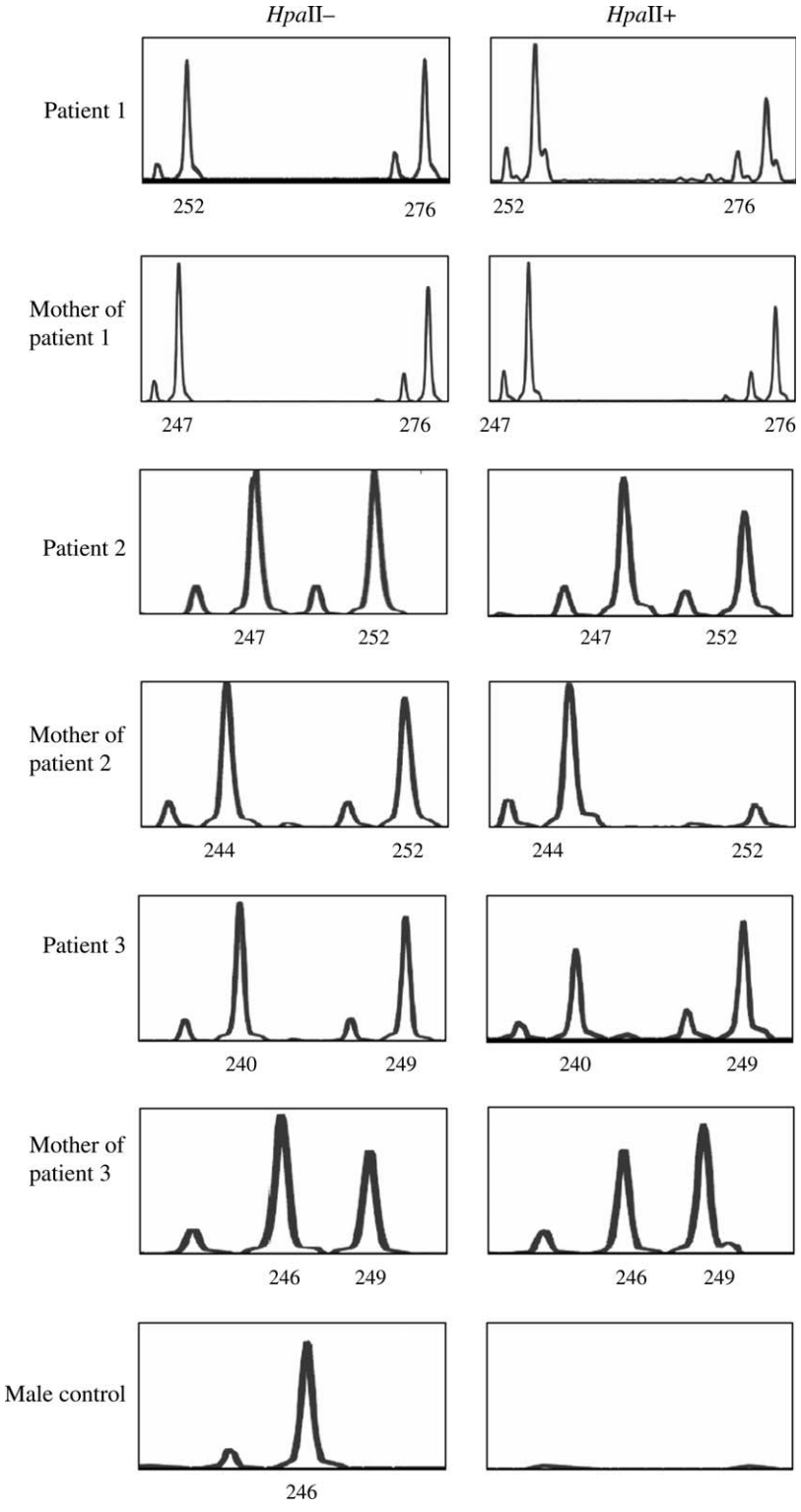

Fig. 4. X chromosome inactivation analysis. '*Hpa*II−' indicates no prior *Hpa*II digestion, and '*Hpa*II+' indicates prior *Hpa*II digestion.

never been described. It is the second splice site mutation ever reported in this gene with the first being c.720-2A>T in intron 8 (2).

Two novel heterozygous mutations in *PORCN* were identified in patient 2. The c.737_738insA in exon 9, expected to result in changing the serine to glutamate at position 247, subsequent changes of 67 amino acids and truncation at codon 315 (p.S247EfsX315), is highly likely pathogenic. Although her mother did not harbor the mutation, we could not definitely confirm its *de novo* nature because of unavailability of her father's DNA sample. The other DNA change found in patient 2 was a c.682C>T transition

in exon 6. Several lines of evidence support its possible etiologic role including being a non-synonymous change expected to result in conversion of an arginine to a cysteine (p.R228C). It has not been reported to be a polymorphism in NCBI SNP Database (http://www.ncbi.nlm.nih.gov/projects/SNP/), Ensembl (http://www.ensembl.org/index.html), PupaSUITE/PupaSNP (http://pupasuite.bioinfo.cipf.es/), and UniProt (http://www.expasy.uniprot.org/index.shtml). In addition, it was not detected in 162 ethnic-matched control X chromosomes. Using PolyPhen (http://coot.embl.de/PolyPhen/), this variant is predicted to be possibly damaging. On the contrary, there are some evidences suggesting that it is not pathogenic. First, the c.682C>T mutation that was detected on the same allele as the c.737_738insA in the patient was found heterozygously in her clinically unaffected mother. Second, the arginine at codon 228 is conserved in chimpanzee, dog, house mouse, and African frog but not in chicken. Finally, this position is not located in a known functional domain. The significance of this change will be further elucidated if functional studies are available.

The c.1094G>A mutation identified in patient 3 is also novel. It is expected to result in an arginine to glutamine substitution at position 365 (p.R365Q). This nucleotide substitution was next to the one that has been previously described in a male patient with Goltz syndrome (the c.1093C>G transition resulting in an amino acid substitution R365G) (3). This finding confirmed a significant role of this critical residue.

X chromosome inactivation analysis showed that all three patients who were sporadic cases had a random X inactivation pattern. Our data also showed that the heights of the signals of all three mutations were similar to those of the wild type. These findings cannot ascertain the origin of the mutations, either germ line or post-zygotic.

X inactivation patterns of the mothers of patients 1 and 3 were random but that of patient 2's mother was partially skewed with a ratio of 82:18 (Fig. 4). She was clinically unaffected but had the DNA change, the pathogenic role of which is inconclusive. Although the real explanation for the skewed pattern of X chromosome inactivation in PBL-derived DNA in this mother remains elusive, one possibility is that she was heterozygous for a pathogenic mutation in any X-linked gene, including the possibly pathogenic *PORCN* c.682C>T. With the favorable X inactivation, she remained unaffected.

Although human PORCN has not been well characterized biochemically, the homology of human *PORCN* to the porcupine gene family in *Drosophila* and in mice suggests that the human gene encodes an *O*-acyltransferase. It catalyses cysteine *N*-palmitoylation and serine *O*-acylation in the endoplasmic reticulum, allowing membrane targeting and secretion of several Wnt proteins (7, 8). Characterization and expansion of the *PORCN* mutational spectrum would help identify important functional domains of the protein.

In summary, we reported three unrelated Thai female sporadic cases of FDH. One of them had unilateral athelia, which has never been described in FDH. Novel pathogenic mutations, c.373+1G>A, c.737_738insA and c.1094G>A, were identified. Another novel variant, c.682C>T, with inconclusive role was also detected. All patients had a random X inactivation. This study demonstrates that *PORCN* is the gene responsible for FDH across different populations and expands the genotypic spectrum of *PORCN* mutations.

Acknowledgements

We are grateful to the affected individuals and their families for their participation. This study was supported by a Research Unit Grant from Chulalongkorn University, the National Center for Genetic Engineering and Biotechnology, the Thailand Research Fund and the Toyota Thailand Foundation.

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