Identification of Two Novel Missense Mutations (p.R1221C and p.R1357W) in the ABCC6 (MRP6) Gene in a Japanese Patient with Pseudoxanthoma Elasticum (PXE)

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Abstract

Pseudoxanthoma elasticum (PXE) is a rare, inherited, systemic disease of elastic tissue that in particular affects the skin, eyes, and cardiovascular system. Recently, the *ABCC6* (*MRP6*) gene was found to cause PXE. A defective type of *ABCC6* gene (16p13.1) was determined in two Japanese patients with PXE. In order to determine whether these patients have a defect in *ABCC6* gene, we examined each of 31 exons and flanking intron sequences by PCR methods (SSCP screening and direct sequencing). We found two novel missense variants in exon 26 and 29 in a compound heterozygous state in the first patient. One is a missense mutation (c.3661C>T; p.R1221C) in exon 26 and the other is a missense mutation (c.4069C>T; p.R1357W) in exon 29. These mutations have not been detected in our control panel of 200 alleles. To our knowledge, this is the first report of mutation identification in the *ABCC6* gene in Japanese PXE patients. The second patient was homozygous for 2542_2543delG in *ABCC6* gene and heterozygous for 6 kb deletion of *LDL-R* gene. This case is the first report of a genetically confirmed case of double mutations both in PXE and FH loci.

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Key words: pseudoxanthoma elasticum, PXE, ATP binding cassette transporter, ABCC6, MRP6, membrane transporter proteins, calcification

Introduction

Pseudoxanthoma elasticum (PXE [OMIM 177850 and OMIM 264800]) is a heritable disorder of the connective tissue characterized by progressive calcification of elastic fibers in skin, retina, and the cardiovascular system. PXE is usually inherited as an autosomal recessive trait, but examples of autosomal dominant and sporadic forms of PXE have been reported. The prevalence of the disease in the general population ranges between 1/70,000 and 1/160,000 (1).

Recently, mutations in the *ABCC6* (*MRP6*) gene have been identified to be responsible for PXE (2, 3). The precise function of ABCC6 is currently unknown. The *ABCC6* gene is predominantly expressed in both the liver and kidney (4), although these two organs were not thought to be involved in the development of PXE.

Le Saux et al demonstrated that the nonsense mutation p.R1141X and a large deletion spanning from exon 23 to exon 29 were relatively common mutations in a cohort of 122 patients from the United Kingdom, the United States, South Africa, Italy, Germany, and Belgium (5). However with the exception of one case report (6), to date there have been no studies that analyzed mutations in the *ABCC6* gene in PXE patients of Asian origin.

Here, we describe two unrelated Japanese patients affected by PXE and their family members. We provide the result of our mutational analysis of the *ABCC6* gene and discuss the significance of the mutations identified.

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Patients and methods

Case presentations

Case 1

Case 1 was a 23-year-old female, with yellow-colored skin lesions since her early teens. She experienced shortness of breath during the previous two years. She was diagnosed with PXE by skin biopsy with Elastica van Gieson staining and presented opthalmological manifestations. Cardiac catheterization revealed coronary stenosis; two 90% stenoses were present in the right coronary artery (RCA), the left circumflex coronary artery (LCX) had a 95% stenosis (Fig. 1A). There was no family history of PXE and her parents marriage was not consanguineous (Fig. 2).

Case 2

Case 2 was a 75-year-old female. She was clinically diagnosed with PXE associated with familial hypercholesterolemia (FH), and reported by us (7) at the age of 53. She was hypertensive (210/110 mmHg) and had a history of cerebral bleeding resulting in right hemiparesis at the age of 55. Yellow-colored skin lesions were observed on her neck, axilla, umbilical and inguinal regions. Umbilical skin biopsy revealed characteristic fragmentation, clumping and calcification of elastic tissue by Elastica van Gieson and Kossa stain. She did not have any ophthalmological manifestation. Laboratory data showed TC of 7.88 mmol/*l* (305 mg/dl), TG of 1.57 mmol/*l* (139 mg/dl) and HDL-C of 0.80 mmol/*l* (31 mg/dl). Achilles tendon thickness by X-ray examination was 16 mm (normal thickness <9 mm). Her second son also had hypercholesterolemia (TC: 8.84 mmol/*l*; 342 mg/dl) and Achilles tendon xanthoma. The electrocardiogram (ECG) of this proband showed horizontal ST segment depression in I, II, $_{a}V_{L}$, V_{3-6} leads. Remarkable calcification was apparent from the ascending aorta to the femoral arteries as well as the coronary arteries by X-ray examination and computed tomography (Fig. 1B, C). Her father, elder brother and the third son died from heart disease at 58, 53 and 22 years old, respectively. The proband and proband's second son had hypercholesterolemia, although the other members did not. There was no family history of PXE (Fig. 2).

Mutation detection

Written informed consent was obtained from all participants. The Institutional Review Board of each institution had approved this study. Blood samples were collected from the patients and 100 unaffected and unrelated Japanese control individuals. Genomic DNA was isolated from peripheral blood leukocytes according to standard procedures and was used as a template for polymerase chain reaction (PCR). The sequence information was obtained from the published sequence of human chromosome 16 BAC clone A-962B4 (GenBank Accession No. U91318) and the intron-exon borders for the 31 exons in *ABCC6* were inferred by comparison with the published cDNA sequence (GenBank Accession No. AF076622, the primer sequences are available in Table 1). PCR products were 142–368 bp and included complete intron/exon boundaries and were screened by SSCP (single-strand conformation polymorphism) analysis (8). Sequence analysis was carried out with the dideoxynucleotide chain termination method, using a Thermo sequenase II (Amersham Pharmatica Biotech, Cleveland, OH, USA) in ABI 310 automated DNA sequencer (PerkinElmer, Foster City, CA, USA). The mutations found in the *ABCC6* gene were further confirmed by the PCR-restriction fragment length polymorphism (RFLP) method.

Screening for the deletion ABCC6del23–29

We screened for the 16.5-kb Alu-mediated deletion (ABCC6del23-29) that extends from intron 22 to intron 29 and that has been reported to be a relatively common in Caucasian PXE patients using the method previously described (9).

Results

Two novel mutations of the *ABCC6* gene were identified in case 1. One was a heterozygous missense mutation (c.3661C>T; p.R1221C) in exon 26 and the other was a heterozygous missense mutation (c.4096C>T; p.R1357W) in exon 29 of the *ABCC6* gene. The c.3661C>T mutation predicted the creation of a novel recognition site for *TspE* I and the loss of a *Taq* I restriction site. The variant c.4096C>T in exon 29 results in the gain of a novel *Mae* II restriction endonuclease recognition site and in the loss of a *Acc* II restriction endonuclease recognition site.

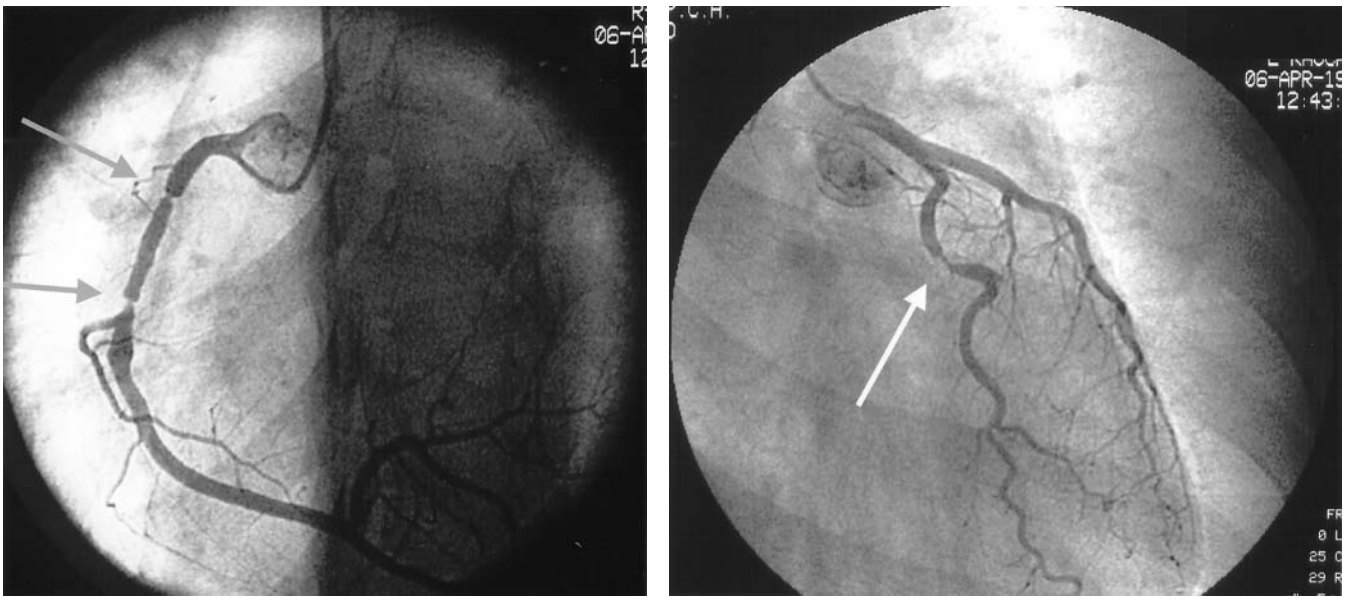
Case 2 was homozygous for the deletion 2542_2543delG in exon 19 of *ABCC6* and heterozygous for a 6 kb deletion (FH-Tonami-1) in the LDL receptor gene (10). This is the first reported case of the molecular diagnosis of mutations in both the PXE locus and the FH locus in a patient affected with both diseases. The second son of case 2 was heterozygous for 2542_2543delG in *ABCC6* and heterozygous for the deletion FH-Tonami-1.

We have found neither the nonsense variant p.R1141X nor the large deletion ABCC6del23–29 in our patients.

Discussion

ABCC6 belongs to the ABC (ATP binding cassette) gene subfamily C, which includes *ABCC1-5*, *CFTR*, *ABCC8*, and *ABCC9* (encoding the sulfonylurea receptor) (11). *ABCC6* consists of 31 exons spanning ~75 kb of DNA and encodes a 165-kD transmembrane protein. The ABCC6 protein is predicted to contain 17 membrane-spanning helices grouped into 3 transmembrane domains. Like other ABC transporters, the protein contains two intracellular nucleotide binding domains (NBD1 and NBD2). Each NBD has conserved Walker A and B motifs that are critical for ATPase function (11).

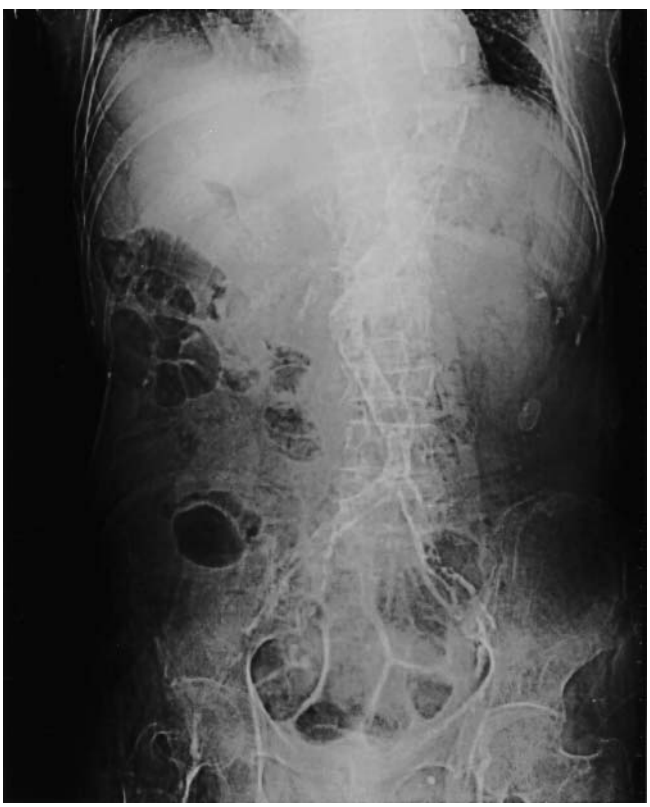
In the present study, two novel missense mutations, p.R1221C and p.R1357W, were found in a young female



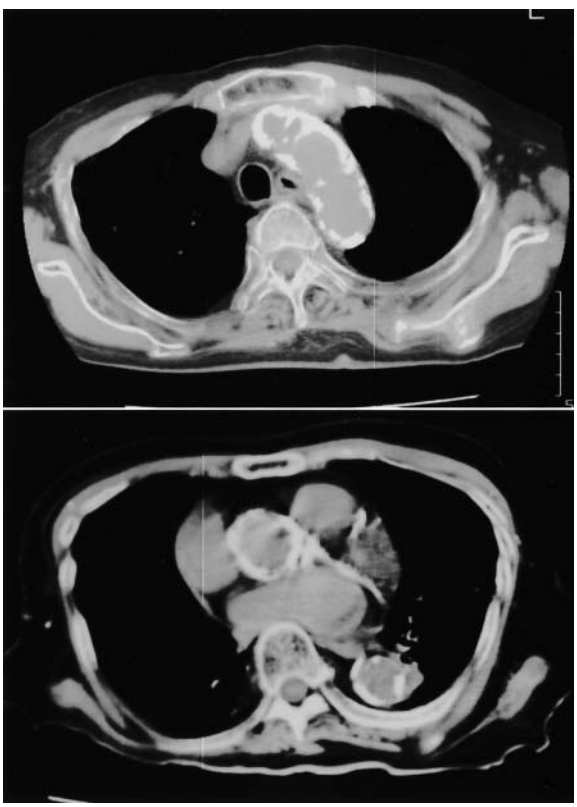
Right coronary artery

Left coronary artery

A



B



C

Figure 1. Phenotypic characteristics of PXE. A) Cardiac catheterization revealed that case 1 had severe stenosis (arrow) in both coronary arteries. B) X-ray image of case 2 showed that remarkable calcification was apparent from the ascending aorta to the femoral arteries. C) Severe calcification was revealed by computed tomography in the coronary arteries as well as aorta (case 2).

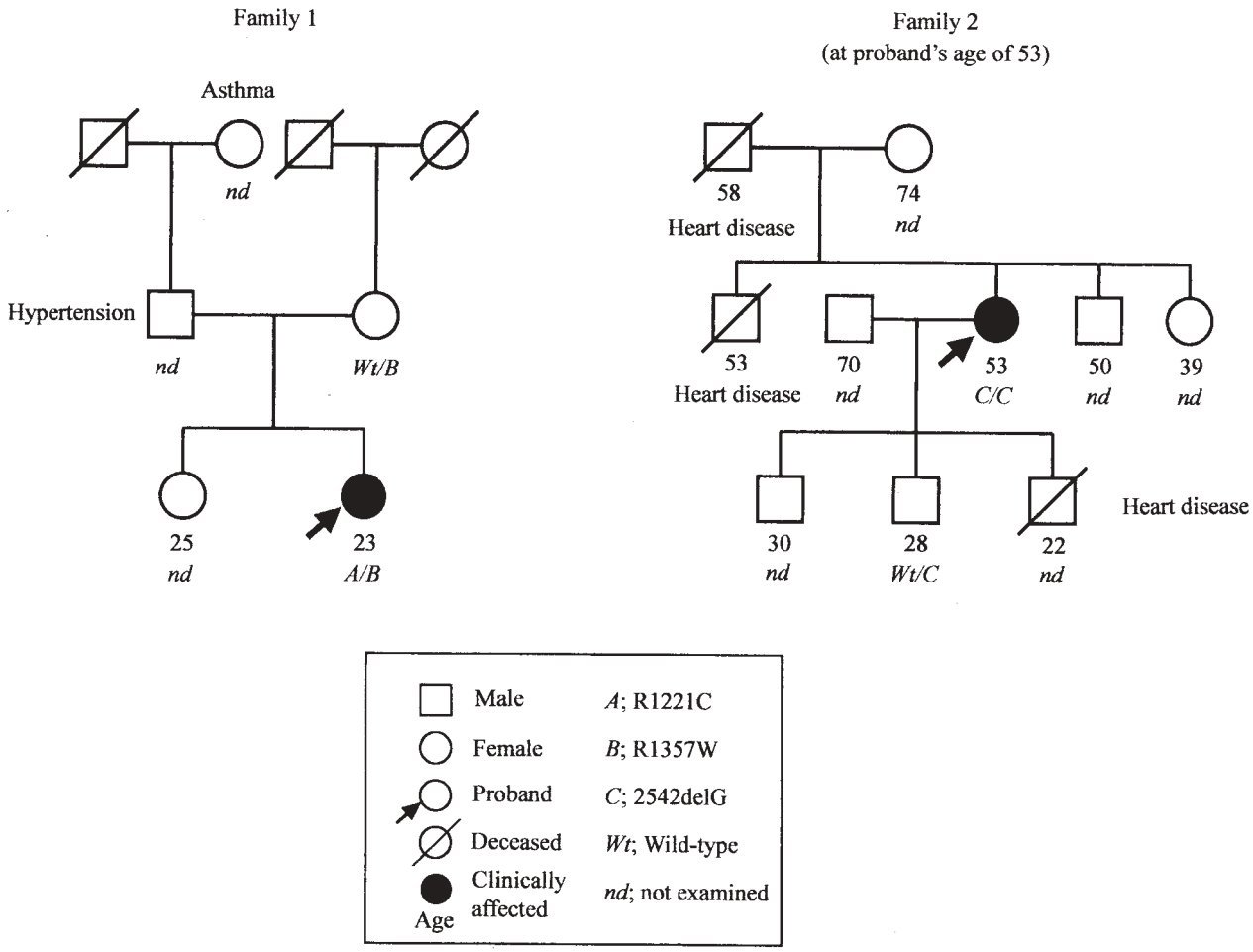


Figure 2. Pedigrees of two patients with PXE. (Family 1) In the family of the case 1, her mother was revealed to be heterozygous for p.R1357W. Her father has not been examined yet. (Family 2) The pedigree of case 2 at proband's age of 53. Her father, elder brother and the third son died of heart disease at 58, 53 and 22 years old, respectively. The proband and proband's second son showed hypercholesterolemia, although the other members did not.

patient who presented severe coronary stenosis. Arginines at positions 1221 and 1357 in the ABCC6 protein represent conserved residues among human ABCC1 (MRP1), ABCC2 (MRP2) and ABCC6 (MRP6) (4). The nonconservative amino acid substitutions found in case 1 were not detected in 100 clinically unaffected, unrelated control individuals. Therefore, we considered these two variants to be disease-causing. p.R1221C and p.R1357W are novel disease-causing mutations in the ABCC6 gene that have not been previously reported.

p.R1221C is located between the 17th transmembrane helix and NBD2. p.R1357W was found in NBD2. *In vitro* transport studies with three PXE-causing mutants (p.V1298F, p.G1302R and p.G1321S) within NBD2, that had been expressed in Sf9 cells, showed markedly reduced transport activities (12). We would expect a similar effect for the p.R1357W. However, for other mutations such as p.R1221C, no such information is currently available.

A small deletion, 2542_2543delG is located C-terminally of NBD1. 2542_2543delG was predicted to result in premature termination of translation. Premature termination mutations frequently result in the nonsense-mediated decay of (NMD) of mutant mRNA products and significantly reduce mutant transcript levels (13). Therefore, the 2542_2543delG mutation could result in NMD.

The small deletion 2542_2543delG has previously been reported in only one case (14). The frequency of this deletion and the ethnicity of the patient(s) carrying this mutation were not reported.

PXE was first described as a sporadic disorder, but both autosomal recessive and autosomal dominant inheritances have been reported (1, 3, 15). ABCC6 mutations have been reported in three families with apparently dominant PXE. In two families, the recurrent recessive mutation p.R1141X was identified in affected patients (3). In a third family, the three affected siblings were heterozygous for the maternal

Table 1. Oligonucleotide Sequence

Primer	Name	Sequence	Product size (bp)
Exon1			
	E1F	5′-GAATTTGGGGGTCTCTCCTC-3′	191
	E1R	5′-GCCCAGAGACTTAGCGACAG-3′	
Exon2			
	E2F	5′-CGAACATTGCCTGGTTCC-3′	268
	E2R	5′-CCCTGCCTTGTACCATCCTA-3′	
Exon3			
	E3F	5′-CTCCAGACTGAAGGCATCAT-3′	207
	E3R	5′-CCAGTTTGCTGTGACCTCTCT-3′	
Exon4			
	E4F	5′-ATGAGCCACCATTTTGGTTT-3′	206
	E4R	5′-CTAAGGGGCCTCCCTGACT-3′	
Exon5			
	E5F	5′-GGAACAGGAATGAGGTTGGA-3′	212
	E5R	5′-CTGAGCACCCTCCTCTGTCT-3′	
Exon6			
	E6F	5′-GGGAATCAGAGCAGCAAATG-3′	180
	E6R	5′-GTCTTCCTACCCTTGCCACA-3′	
Exon7			
	E7F	5′-TTCTTGACCTCCACCCACTT-3′	241
	E7R	5′-ACCCAGGGTCACACAGCTAC-3′	
Exon8			
	E8F	5′-GAGACCACCCACCTTAGCAG-3′	303
	E8R	5′-GCTGGCGGCTGAGAGTATAA-3′	
Exon9			
	E9F	5′-CCCGCTCAGTGATACTGCTT-3′	271
	E9R	5′-CAGCTGTACCTTCTCCCTCCT-3′	
Exon10			
	E10F	5′-ACTCCGTTCAAATCCCGTCT-3′	239
	E10R	5′-GGCCTCCCCACTTTACTTCT-3′	
Exon11			
	E11F	5′-GTGGCTTCCTCCCTACTTCC-3′	200
	E11R	5′-CTCTGAGAGCTGGGCTCCT-3′	
Exon12			
	E12F	5′-CAGTGCTGCTCAGCATAGAGA-3′	306
	E12R	5′-GTCAGGGTGCAGGGAAGAAT-3′	
Exon13			
	E13F	5′-GAAGCTGGAGCCAGGTGTAG-3′	263
	E13R	5′-TATCCATGCTTGCGTGTCTC-3′	
Exon14			
	E14F	5′-CAGTACTGATGCTGGCTTGC-3′	217
	E14R	5′-CTCTTCTTGCTGGGTGACCT-3′	
Exon15			
	E15F	5′-GGCTGGTTACTACGGGTGTC-3′	142
	E15R	5′-CAGGGGTCTCCTGTAAATGG-3′	
Exon16			
	E16F	5′-GATGGGGACATCCTAGCAGA-3′	198
	E16R	5′-CAAGGTCATGTCTCCCCTCT-3′	
Exon17			
	E17F	5′-TGTCTCCCTGTCCCAAAAAG-3′	250
	E17R	5′-CATCATCCTCCTGTGACCAA-3′	
Exon18			
	E18F	5′-AAGTGCTTCCTCTGCCTTTG-3′	250
	E18R	5′-CAGGCCACTGTTCCTTTTGT-3′	
Exon19			
	E19F	5′-GAATCAGCAAAGCCCACCTA-3′	269
	E19R	5′-TGTTGGGATTACAGGCATGA-3′	
Exon20			
	E20F	5′-AGCCTGTGCCCTTCTGAGT-3′	200
	E20R	5′-AGAGCGGTTAAGGCCACATA-3′	
Exon21			
	E21F	5′-ACATTTGGTGGGAGGACTTG-3′	246
	E21R	5′-CCCACCATTGGGAGAGATAC-3′	
Exon22			
	E22F	5′-GATGAGGAGGGCAGGTGAG-3′	256
	E22R	5′-CCTCTCCCTCATGTGTGCTA-3′	
Exon23			
	E23F	5′-CCTCCCTGACCTCTCCGTA-3′	368
	E23R	5′-TCCAGCCCTCATGCTCTTAC-3′	
Exon24			
	E24F	5′-CTGCCCTGGCTCTTCCTAC-3′	251
	E24R	5′-CTCCTCTTCCCTCTCCCATC-3′	
Exon25			
	E25F	5′-CCTCTGTCTGTCCCTCAAGC-3′	205
	E25R	5′-TAACCACTCACCCTGCTGTC-3′	
Exon26			
	E26F	5′-GATGTCAACAGGGACCCATT-3′	200
	E26R	5′-CCAGAGAGGCTTTCTTGCAC-3′	
Exon27			
	E27F	5′-GTCCTTTGGCCTAAACTCCA-3′	221
	E27R	5′-ACTCAGTTTCCCCTCCTGCT-3′	
Exon28			
	E28F	5′-ACCATGCCTCCCATCTTTG-3′	260
	E28R	5′-CCAATAAATGCCCACAAACC-3′	
Exon29			
	E29F	5′-ATTTCCTGAAGGCCCTTGG-3′	303
	E29R	5′-AAAGATGGGAGGCATGGTG-3′	
Exon30			
	E30F	5′-GGCTGCTGTGAGGTCAGG-3′	258
	E30R	5′-CCAGCTAATTGTCCCAATCG-3′	
Exon31α*			
	E31Fα	5′-TGGGGTACCAAGTACACGAA-3′	360
	E31Rα	5′-AGACCTGTGTTTGCTCTCTGG-3′	
Exon31β*			
	E31Fβ	5′-GCCACTTTCTCTGCCATTTT-3′	286
	E31Rβ	5′-GCCTCTCTGTCTCCCTCTCC-3′	
ABCC6del23-29			
	DEL-1	5′-GGAATACTCAAGGCGAATGG-3′	652
	DEL-2	5′-TCTTGAAGCAGCAGTGAGTC-3′	552
	DEL-3	5′-TTGAGCAGGCTGACTGTAGG-3′	

*Exon 31α primer set can amplify the first half of exon 31, and Exon 31β primer set can amplify the latter half.

inherited p.R1495C mutation. Autosomal recessive inheritance could not be excluded in this family, however, as the three patients inherited the same paternal allele (16). In fact, no molecular evidence for autosomal dominant inheritance has been demonstrated at present, although some of the heterozygous carriers may demonstrate minimal manifestation of the disease (17). Taken together, these data support a unique recessive mode of inheritance in PXE (18).

Ohtani and Furukawa reported a very preliminary mutational analysis of a Japanese patient (6). They examined only 5 out of the 31 exons of *ABCC6* and failed to find any mutation responsible for PXE. They did not examine the exons in which we identified as disease-causing mutations in our patients that include exon 19 [2542_2543delG], exon 26 [p.R1221C], and exon 29 [p.R1357W].

In summary, we have performed mutational analysis for

all 31 exons of ABCC6 gene in Japanese patients with PXE. Two novel mutations, both affecting highly conserved amino acids and one previously reported mutation responsible for PXE have been identified. To the best of our knowledge, this is the first report of mutation identification in the ABCC6 gene in Japanese PXE patients in the English language literature. However, one of the major limitations of this study is that only restricted family members were analyzed. Because the number of patients in this study was small, further investigations for a nation-wide survey of Japanese patients with PXE are needed to determine, if indeed a different spectrum of private and recurrent mutations in *ABCC6* is responsible for PXE in the Japanese population as compared to the previously studied North American and European populations.

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