Original article

Analysis of clinical symptoms and *ABCC6* mutations in 76 Japanese patients with pseudoxanthoma elasticum

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ABSTRACT

Pseudoxanthoma elasticum (PXE) is a hereditary disease, causing calcification and

degeneration of elastic fibers, which affects the skin, eye, cardiovascular systems, and

gastrointestinal tract. PXE is caused by mutations in the *ABCC6* gene. Neither detailed

nor large-scale analyses have been accomplished in Japanese patients with PXE. We,

therefore, investigated clinical symptoms and *ABCC6* gene mutations in 76 Japanese

patients.

Japanese PXE patients (n = 76) had a significantly lower incidence of vascular lesions

than 505 PXE patients in the Leiden Open Variation Database (LOVD) (38.7% vs.

65.1%, respectively; p = 1.34E-06); however, the incidences of the skin, eye, cardiac,

and gastrointestinal lesion symptoms were not significantly different. Symptoms

severity scores for skin, eye, and vascular lesions, calculated using the Phenodex™

system, were significantly lower in Japanese PXE patients than in LOVD PXE patients.

Genetic analysis revealed three nonsense, four frameshift, one exon deletion, and 13

missense mutations in *ABCC6* in 73 patients; however, we were unable to detect

pathogenic mutations in three patients. Frequent mutations differed between Japanese

and LOVD PXE patients. In Japanese PXE patients, the top five mutations accounted

for more than 60% of all pathogenic changes, suggesting the presence of founder effects.

Consistent with previous reports, no obvious correlations between genotypes and phenotypes were identified in this study.

In conclusion, we consider that the milder clinical phenotypes, observed even in older Japanese PXE patients, could be attributed to environmental factors such as dietary habits and lifestyle, as well as genetic background.

INTRODUCTION

Pseudoxanthoma elasticum (PXE; OMIM #264800) is an autosomal recessive disorder

characterized by aggregation, fragmentation, and calcification of elastic fibers. PXE

primarily affects organs and tissues that are rich in elastic fibers, such as the skin and

mucosa, retina, blood vessels, and gastrointestinal tract [1]. Typical cutaneous

manifestations are yellow-white papules or wide yellowish skin folds and redundant

skin mainly in the neck, axillae, cubital fossa, papliteal fossa, and periumbilical and

inguinal regions. In the eye, angioid streaks and peau d'orange are caused by rupture of

Bruch's membrane and often lead to subretinal hemorrhages, choroidal

neovascularization, and visual loss [2]. Patients have an increased risk of ischemic

complications such as coldness of the limbs, intermittent claudication, angina pectoris,

and myocardial infarction or cerebral infarction [3].

PXE is caused by mutations in *ABCC6*, a gene that encodes a transmembrane protein

(ABCC6), thought to be a transporter [4-8]. For many years, the function of the ABCC6

molecule was unknown; however it was recently demonstrated that ABCC6-mediated

ATP release from the liver is the main source of plasma pyrophosphate, and that plasma

pyrophosphate concentrations are lower in PXE patients than in healthy individuals [9].

Pyrophosphate is a key regulator of ectopic mineralization that acts by inhibiting hydroxyapatite crystal growth [10]. Therefore, the ectopic calcification observed in patients with PXE may be caused by abnormal pyrophosphate metabolism.

Large-scale studies of gene mutations in PXE have been reported from Europe [11-13], North America [12], China [14], and South Africa [12,15]. In Japan, until recently, there was only one report of *ABCC6* mutations in patients with angioid streaks [16]. We have since reported cases of *ABCC6* gene mutations in Japanese patients with PXE or PXE-like patients [17-19].

In this report, we investigated symptoms, their severity, and complications as well as characterizing *ABCC6* gene mutations, in 76 Japanese patients with PXE by comparison with 505 patients registered in the Leiden Open Variation Database (LOVD), (http://www.ncbi.nlm.nih.gov/lovd/variants.php?select_db=ABCC6&action=view_all).

METHODS

PXE patients and diagnosis

Diagnosis of PXE was made using our criteria, which are largely based on those of

Lebwohl and Promp [20,21]. According to these criteria, 'definite PXE' is defined by

typical abnormalities in both the skin and the eye, or either of these in association with

an *ABCC6* gene mutation. Positive skin findings are defined as either typical cutaneous

lesions or degeneration and/or calcification of elastic fibers, while positive eye findings

consist of either angioid streaks or peau d'orange. 'Probable PXE' is defined as positive

for either skin or eye findings as described above, without gene mutations in *ABCC6*. In

this study, gene analysis and investigation were performed of clinical phenotypes in

patients with PXE who were diagnosed between 2010 and 2014. This study was

approved by the ethical committee of the Graduate School of Biomedical Sciences,

Nagasaki University and written informed consent was obtained from all 76 patients in

Nagasaki University, Kyoto University, Osaka Medical College, Osaka University, and

Gunma University.

Scores of phenotype severity

The severity of symptoms was estimated using the Phenodex™ system, proposed by

PXE international (https://www.pxe.org). Phenodex™ scores for skin (S), eye (E),

gastrointestinal tract (G), vascular (V), and cardiac (C) symptoms are detailed in Table

S1.

Derm-score

Our original system, of 'derm-score', was used to evaluate the distribution of skin and

mucosal lesions [22]. Briefly, the presence or absence of the lesions in the neck, axillae,

cubital fossa, periumbilical region, inguinal regions, and oral mucosa was converted to

a binary score (presence = 1, absence = 0), irrespective of their severity. For each

patient, scores from six sites were summed to yield a total distribution score

(derm-score). Higher derm-scores and the presence of oral mucosal lesions are

associated with cardiovascular disease in Japanese PXE patients [22]. In this study,

cardiovascular diseases included intermittent claudication, angina pectoris, myocardial

infarction, and cerebral infarction.

Genome sequence of ABCC6 gene

DNA was extracted from blood using a DNeasy® Blood & Tissue Kit (Qiagen, Hilden, Germany). Each exon of *ABCC6* was amplified with primers designed using Primer 3 software (ver 0.4.0) (Table S2). Two *ABCC6* pseudogenes, *ABCC6Φ1* (Entrez Gene ID 653190) and *ABCC6Φ2* (Entrez Gene ID 730013), contain DNA sequences homologous to exons 1–4 and exons 1–9 of *ABCC6* [23,24], respectively, which hampers mutational analysis of the *ABCC6* gene [25]. To exclude these two pseudogenes, primers specific to *ABCC6* exons 1–9 were utilized according to a previous report [24].

For PCR reaction mixtures contained 1–5 ng of DNA, 0.25 pmol of each forward and reverse primer, 0.5 µl of KOD-FX (TOYOBO, Osaka, Japan), 12.5 µl of PCR buffer, 2 mM dNTPs, and distilled H_2O in a final volume of 25 µl. Samples were denatured at 94°C for 1 min, followed by 30 cycles of 95°C for 10 s, 55°C or 58°C for 30 s, and 72°C for 1 min. A final extension cycle of 72°C for 1 min was performed. An aliquot of each PCR product was electrophoresed on a 2% agarose gel. The DNA sequencing was performed using a Big-Dye® Terminator v3.1 Cycle Sequencing Kit (Applied

Biosystems, Foster city, CA, USA) and an ABI PRISM® 3130 Genetic Analyzer

(Applied Biosystems).

Genome sequence of ENPP1 gene

Genome sequence of *ENPP1* gene was performed for three patients who had no *ABCC6*

gene mutations. Primers of *ENPP1* were designed using Primer3Plus

(http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). Twenty-five primer

pairs were used for amplification and sequences of the 25 exons of *ENPP1* (RefSeq

accession number NG_008206.1). Primer sequences are available upon request. For

PCR reaction and DNA sequencing, they were performed as same as those of *ABCC6*.

Exon deletion detection

To evaluate exon deletions, multiplex ligation-dependent probe amplification (MLPA)

analysis for the *ABCC6* gene was performed using the MLPA SALSA® kit P092

(MRC-Holland, Amsterdam, Netherlands), according to the manufacturer's protocol,

containing exons 2, 4, 5, 7, 8, 9, 10, 11, 12, 13, 14, 15, 17, 18, 21, 22, 23, 24, 25, 26, 27,

28, and 30 of whole 31 exons. Products were analyzed using an ABI PRISM® 3130

Genetic Analyzer (Applied Biosystems) and semi-quantified using GeneMapper®

(Applied Biosystems). When the value of an exon signal was < 60% of that of the mean

of normal controls, it was determined to represent monoallelic exon deletion; when the

signal was < 1%, it was determined to represent biallelic exon deletion.

Statistical analyses

The incidence of Japanese PXE patients and those registered in LOVD

(http://www.ncbi.nlm.nih.gov/lovd/variants.php?select_db=ABCC6&action=view_all)

were analyzed by the Fischer's exact test. The clinical severities of Japanese PXE

patients and those in LOVD were analyzed by the Student's t-test. Associations of gene

mutations with skin lesions, angioid streaks, and cardiovascular diseases were estimated

using Phenodex™ scores. Gene mutations were categorized into groups as follows:

Group A, two nonsense mutations, or two frameshift mutations, or one nonsense and

one frameshift mutation; Group B, only one nonsense or frameshift mutation; and

Group C, two missense mutations. Correlations between genotypes and organ-specific

symptoms, as well as symptom severity, were analyzed by the Student's t-test, Fisher's

exact test and Kruskal-Wallis test using STATA software version 13.1 (Stata Corp,

Collage Station, TX, USA).

RESULTS

Clinical symptoms of Japanese PXE patients

The profiles of 76 Japanese PXE patients included in this study are provided in Table S3.

The mean age of the patients was 53.9 years (range, 4–88 years), which was

significantly older than that of 505 PXE patients registered in the LOVD (mean age,

45.7 years; p = 1.76E-05). As shown in Table 1, 25 patients were male (33%) and 51

were female (67%). Among skin, eye, vascular, cardiac, and gastrointestinal lesions,

only vascular lesions were significantly less frequent in Japanese PXE patients than in

LOVD PXE patients, with incidences in each group of 38.7% and 65.1%, respectively

(p = 1.34E-06). No significant differences were observed between Japanese and LOVD

PXE patients in the incidences of skin (96.1% vs. 93.9%, respectively; p = 0.47), eye

(80.0% vs. 88.1%, respectively; p = 0.08), cardiac (24.7% vs. 24.3%, respectively; p =

0.95), or gastrointestinal (5.7% vs. 9.7%, respectively; p = 0.30) lesions (Table 1).

We compared the severities of the clinical symptoms of Japanese PXE patients to those

registered in the LOVD by evaluating average Phenodex™ scores (Table 2). There were

significant differences in the severity scores between Japanese and LOVD PXE patients

of skin (0.97 vs. 2.17, respectively; p = 3.89E-58), eye (1.83 vs. 2.29, respectively; p =

6.06E-04), and vascular (0.53 vs. 1.26, respectively; p = 7.29E-09) lesions, with

Japanese scores significantly lower in each case. No significant differences were

observed in severity scores between Japanese and LOVD PXE patients for cardiac (0.31

vs. 0.30, respectively; p = 0.82), or gastrointestinal (0.06 vs. 0.09, respectively; p =

0.39) lesions.

Gene analysis

Mutations were identified in 127 of 152 alleles in the 76 patients; however, three of 76

patients (#74–76) had no detectable mutations (Table S3). There were 22 patients with

homozygous mutations, 32 patients with compound heterozygous mutations, and 19

patients with one heterozygous mutation. Based on known *ABCC6* single nucleotide

polymorphisms (SNPs) (http://www.ncbi.nlm.nih.gov/snp/?term=ABCC6), we found

113 non-pathogenic single nucleotide variants in the 76 Japanese PXE patients and

these were excluded from further analysis.

As shown in Table 3, the five most frequent mutations were c.2542delG, p.V848CfsX83

(n = 34, 22.4%); c.1132C>T, p.Q378X (n = 30, 19.7%); deletion of exons 2 and 4 (n =

15, 9.9%); c.595C>T, p.Q199X (n = 11, 7.2%); and c.1256G>A, p.R419Q (n = 9, 5.9%).

Among the list of mutations in 505 patients with PXE in the LOVD, the most frequent

five mutations were c.3421C>T, p.R1141X, (n = 125, 12.4%); deletion of exons 23–29

(n = 67, 6.6%); c.4015C>T, p.R1339C (n = 44, 4.4%); c.3490C>T, p.R1164X (n = 21,

2.1%); and c.3413G>A, p.R1138Q (n = 12, 1.2%). In total, the five most frequent

mutations in Japan occurred in 65.1% of cases, whereas for the 505 LOVD cases, they

occurred in only 26.7%. These observations suggest that *ABCC6* mutations in Japanese

PXE patients are derived from a relatively small number of founders, as observed in

South African patients [15,31].

The locations in the ABCC6 protein of the amino acids encoded by all 13 missense

mutations identified in this study are shown in Figure 1. These include six mutations

previously reported as pathogenic, namely, c.1987G>A, p.G663S; c.2477T>C, p.L826P;

c.3412C>T, p.R1138W; c.3491G>A, p.R1164Q; c.4015C>T, p.R1339C; and c.4069C>T,

p.R1357W [4,7,25,26,27]. Seven novel missense mutations were identified, namely,

c.787G>T, p.A263S; c.1256G>A, p.R419Q; c.1465C>T, p.R489W; c.3107T>C,

p.F1036S; c.3533T>C, p.L1178P; c.4279G>A, p.E1427K; and c.4375G>A, p.R1459H.

In this study, we defined novel pathogenic missense mutations as those with frequencies of < 1% and not registered as non-pathogenic *ABCC6* SNPs (http://www.ncbi.nlm.nih.gov/snp/?term=ABCC6). We examined the incidence of the missense mutations listed in Table 3 with three different data base such as Tohoku University Medical Megabank Organization (http://www.megabank.tohoku.ac.jp/), Human Genetic Variation Database (http://www.genome.med.kyoto-u.ac.jp/SnpDB/), and Exome Aggregation Consortium (http://exac.broadinstitute.org/) and all data bases of normal controls confirmed their frequencies were less than 1%. All missense mutations were located in parts of the gene encoding the intracellular region of ABCC6 and a number of these were closed to the nucleotide-binding folds, suggesting that intracellular conformation is critical for the activity of the ABCC6 molecule.

Functional assays are required to determine pathogenicity of missense mutation. In fact, there were reports studying function of mutated ABCC6 molecules by counting ATPase catalysis activity and by detecting subcellular localization in mouse liver using transfection methods with mutated ABCC6 [32,33]. Furthermore, rescue abilities of missense mutations were analyzed by morpholino oligonucleotides-mediated

knock-down methods of abcc6a with zebrafish [33] . However, in this study, we

analyzed those missense mutations with well-established three mutation prediction

programs such as PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), SIFT

(http://sift.jcvi.org/), and FATHMM (http://fathmm.biocompute.org.uk/). Missense

mutations such as p.R419Q; p.R489W; p.G663S; p.F1036S; p.R1138W; p.L1178P;

p.R1339C; p.R1357W; p.E1427K; p.R1459H were predicted to be probably damaging

and deleterious by all three programs (Table 4). The mutation, p.L826P, was predicted to

be probably damaging by PolyPhen 2, deleterious by SHIFT, and tolerated by FATHMM.

The mutation, p.R1164Q, was predicted to be possibly damaging by PolyPhen 2,

tolerated by SIFT, and deleterious by FATHMM. The mutations, p.A263S, was predicted

to be benign by PolyPhen 2, tolerated by SIFT, and deleterious by FATHMM. Therefore,

we thought these three mutations as 'undetermined pathogenic mutations' (Table 4).

Analysis of deletions in the 5'coding region of ABCC6

Using MLPA, we were able to perform semi-quantitative analyses of each *ABCC6* exon

revealing homozygous deletions of exons 2 and 4 in patients #51–53; however, using

PCR and Taq polymerase KOD-FX (TOYOBO, Osaka, Japan), products of the expected

size were amplified using primer sets for exons 1, 2, and 3/4. DNA sequence analysis of

those products in the patients #51–53 revealed that the PCR products were derived from

the pseudogenes *ABCC6Φ1*, which contains sequences similar to exons 1–9, and

ABCC6Φ2, which contains sequences homologous to exons 1–4. This suggests that

even though the assay employed pseudogene-excluding primers, this enzyme amplified

the pseudogenes in the absence of genuine *ABCC6* gene exons 1–4. By contrast, in a

case with heterozygous deletion of exons 2 and 4, the DNA sequence of PCR products

containing exons 1–4 consisted of only the genuine *ABCC6* gene, with no pseudogene

sequence. Therefore, as previously reported [24], the pseudogene-excluding primers

successfully prevented amplification of pseudogene exons when genuine exons 1–4 of

the *ABCC6* gene were present.

After several trials, we found that TaKaRa Taq™ (Takara Bio Inc., Shiga, Japan),

which has no 3' endonuclease activity, successfully abolished false amplification of

pseudogenes. Using this method, both exons 1 and 3 were found to be homozygously

deleted in patients #51–53 (Figure 2). However, because this simple exon-amplification

method is not proven to be sufficiently reliable for detection of heterozygous deletions,

we chose to use the MLPA method to precisely quantify exon number. MLPA assays of

the *ABCC6* gene revealed heterozygous deletion of exons 2 and 4 in nine further cases

(Table S3).

Genotype-phenotype correlation

We examined correlations between genotypes and phenotypes in Japanese PXE patients.

Patients with only one mutation or no mutations were excluded from the analysis. We

divided 54 patients with two mutations into three groups. Group A (n = 35) had two

nonsense mutations, two frameshift mutations (deletion and insertion), or one nonsense

mutation with one frameshift mutation. Group B (n=10) had one missense mutation in

combination with either a nonsense mutation or a frameshift mutation. Group C (n = 9)

had homozygous or two heterozygous missense mutations. We analyzed the correlation

between gene mutations and the derm-score, and incidences of angioid streaks,

cardiovascular disease, and hypertension for each of the three groups, and found no

evidence of an association for any of the three groups (Table 5). Cardiovascular disease

was found in 17.6% of group A patients, 33.3% of group B patients, and 0% of group C

patients. Although no patients with cardiovascular disease were detected among the nine

patients in group C, the relationship was not statistically significant (p = 0.52,

Kruskal-Wallis test). Furthermore, there were no significant differences among the three

groups (A, B, and C) in the severity of cutaneous, ophthalmic, vascular, cardiac, and

gastrointestinal disease symptoms as measured by the Phenodex™ score (Table 6; p >

0.16, Kurskal-Wallis test). Overall, we did not detect any significant

genotype/phenotype correlations in PXE, consistent with a previous report [12].

DISCUSSION

Clinical symptoms, including skin, eye, and vascular lesions, were significantly less severe in 76 Japanese PXE patients than in 505 LOVD PXE patients. In addition, Japanese PXE patients were older on average than those registered in the LOVD. The higher average age of Japanese PXE patients may be a consequence of their presenting at hospitals later than LOVD patients, due to Japanese PXE patients having less severe symptoms. Moreover, the incidence of vascular lesions was lower among Japanese PXE patients than among those registered in the LOVD. It will be important to standardize the assessment of clinical symptoms in each different country in order to properly understand the clinical situation in PXE, because the severity of PXE symptoms is likely to be influenced by genetic background, as well as environmental factors and lifestyle.

We could not identify any significant associations between mutations and the derm-score or incidences of angioid streaks, cardiovascular disease, or hypertension. No patients with two missense mutations had cardiovascular disease. Although this may suggest that cardiovascular disease is caused by complete loss of ABCC6 function, rather than partial loss of function as a result of missense mutations, the difference was

not statistically significant. Large-scale studies with sufficient statistical power are required to further investigate this finding.

Given the combined data from MLPA (indicating homozygous deletion of exons 2 and 4) and PCR with TaKaRa Taq™ (Takara Bio Inc., indicating homozygous deletion of exons 1 and 3) in patients #51–53, we thought that these patients may have identical deleted regions. To define the deleted region more precisely, whole-genome sequencing (Takara Bio Inc.) was performed on patient #53. The region covering Chr16: 16,312,200–16,693,700, located in the 5' region upstream of *ABCC6* exon 1, was found to have a large deletion in this patient, identified by a drastic reduction (1/30–1/40) in the mean depth of sequence coverage in this region. However, the precise deletion site could not be identified, probably due to the presence of the two pseudogenes.

*ABCC6*Φ1 and *ABCC6*Φ2, which may have interfered with the correct estimation of redundancy reduction in the region of *ABCC6* containing exons 1–4.

In this study, we identified 127 mutations in 76 Japanese PXE patients; two mutations in 54 patients, one mutation in 19 patients, and no mutations in 3 patients. Our detection rate (at least one mutation) in Japanese PXE patients was 96.1% (73/76). This is high

compared with other reports; 77% [11], 66% [12], and 86% [15]. The three patients in

whom we failed to identify any mutations may have deletions of *ABCC6* exons that the

MLPA kit used did not cover, namely, exons 1, 3, 6, 16, 19, 20, 29, and 31. Less

probably, these patients may have mutations in the *ENPP1* gene. Mutations in *ENPP1*

are responsible for generalized arterial calcification of infancy (GACI), which has a

degree of phenotype over lap with PXE [34,35]; however, the severe phenotypes of the

cardiovascular system commonly observed in GACI were not identified in these

patients (Table S3). Although we performed the *ENPP1* gene analysis for three patients,

no mutations were found.

Three patients, #3, #13, and #76, had angioid streaks without typical cutaneous lesions.

By our diagnostic criteria, they were diagnosed as having "probable" PXE. Gene

analysis revealed that two of them had mutations in the *ABCC6* gene (Table S3),

enabling definitive diagnosis as PXE. In this context, mutation analysis is a useful tool

to diagnose early and atypical cases of PXE, which would allow early treatment and

improve the quality of life of PXE patients.

CONFLICT OF INTEREST: The authors have no conflict of interest to declare.

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Table legends

Table 1. Summary of the characteristics of 76 Japanese PXE patients

Total numbers in each column represent numbers of patients with lesions. Patients for whom there were no records were excluded. Mean ages were compared using the Student's t-test. Comparisons of the frequencies of the skin, eye, vascular, cardiac, and gastric lesions were performed using the Fischer's exact test.

Table 2. Severity of clinical symptoms in Japanese and LOVD PXE patients

Mean Phenodex™ scores calculated for skin, eye, vascular, cardiac, and gastric symptoms are listed. Comparisons were performed using the Student's t-test.

Table 3. Summary of *ABCC6* gene mutations in 73 Japanese PXE patients

Table 4. Bioinformatic prediction of pathogenicity of missense mutations.

They were analyzed with three programs such as PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), SIFT (http://sift.jcvi.org/), and FATHMM (http://fathmm.biocompute.org.uk/). The prediction of the consequences of the mutations on the protein function with the score in parentheses was indicated. Ten mutations from the top in this table, were predicted as pathogenic with all three programs. The mutation, p.L826P, was predicted to be tolerated by FATHMM. The mutation, p.R1164Q, was predicted to be tolerated by SIFT. The mutations, p.A263S, was predicted to be benign by PolyPhen 2, tolerated by SIFT. These three mutations were not consistency with three programs. We regarded them as 'undetermined pathogenic mutations'.

Table 5. Assessment of genotype-phenotype correlations

Patients for whom there were no records were excluded from the analyses. All comparisons were calculated using the Kruskal-Wallis test. Group A, two nonsense mutations, two frameshift mutations, or one nonsense and one frameshift mutation; Group B, only one nonsense or frameshift mutation;

Group C, two missense mutations. We used our original system, derm-score,

to evaluate the distribution of skin and mucosal lesions [22]. DS, derm-score;

AS, angioid streaks; CVD, cardiovascular disease; HT, hypertension.

Table 6. Assessment of correlations between genotype and Phenodex™

symptom severity scores

Mean Phenodex™ scores (Table S1;

https://www.pxe.org/pseudoxanthoma-elasticum-research/pseduxoxanthoma

-elasticum-phenodex) for skin, eye, vascular, cardiac, and gastric lesions

were calculated for each genotype group, such as Group A, B, and C (for

details, see Table 5 legend).

Figure legends

Figure 1. Schematic presentation of the ABCC6 protein showing the locations of missense mutations. Six known missense mutations are depicted as red stars and seven novel mutations are depicted as black stars. NBF: nucleotide-binding folds.

Figure 2. Homozygous deletion of *ABCC6* exons 1 and 3 in patients #51–53. MLPA revealed that three PXE patients had homozygous deletions of *ABCC6* exons 2 and 4. Amplification of exon 1 **(a)** and exon 3 **(b)** was performed using TaKaRa Taq™ followed by 1% agarose gel electrophoresis. Expected amplicon size are indicated by arrows. WT: wild type (positive control), M: molecular marker λ Hind III

Supporting information table legend

Table S1. Phenodex™ scoring system

(https://www.pxe.org/pseudoxanthoma-elasticum-research/pseduxoxanthoma-elasticum

-phenodex)

Table S2. Primers used for sequencing of *ABCC6*

Primers for whole exons 1–31 and exons 1–9 were designed to preclude

amplification of *ABCC6* pseudogenes [24].

Table S3. Profiles of PXE patients

Abbreviations: fsX, frameshift; SD, small deletion; NS, nonsense mutation;

LD, large deletion; MS, missense mutation; SI, small insertion.

Clinical severities were estimated using Phenodex™ scores of skin (S), eye

(E), gastric (G), vascular (V), and cardiac (C) lesions. The Phenodex™ scoring

is described in Table S1 (main manuscript).

Table 1.

	Mean age (n)	Sex (male/female)	Patients with lesions, percentage (number/total)				
			Skin	Eye	Vascular	Cardiac	Gastric
Japanese patients	53.9 (76)	25/51	96.1% (73/76)	80.0% (56/70)	38.7% (24/72)	24.7% (18/73)	5.7% (4/70)
LOVD Patients	45.7 (243)	No record	93.9% (246/262)	88.1% (236/268)	65.1% (168/258)	24.3% (63/259)	9.7% (23/237)
p Value	1.76E-05	-	0.47	0.08	1.34E-06	0.95	0.30

Table 2.

	Skin	Eye	Vascular	Cardio	Gastro
Japanese PXE patients	0.97	1.83	0.53	0.31	0.06
Patients in the LOVD	2.17	2.29	1.26	0.30	0.09
p value	3.89E-58	6.06E-04	7.29E-09	0.82	0.39

Table 3.

Variant	Protein	Exon	n (%)	Reference
c.2542delG	p.V848CfsX83	19	34 (22.4)	[21]
c.1132C>T	p.Q378X	9	30 (19.7)	[25]
Ex2,4del	-	2,4	15 (9.9)	This study
c.595C>T	p.Q199X	5	11 (7.2)	[22]
c.1256G>A	p.R419Q	10	9 (5.9)	This study
c.3936insG	p.L1313AfsX8	28	4 (2.6)	This study
c.3412C>T	p.R1138W	24	3 (2.0)	[4]
c.4069C>T	p.R1357W	29	3 (2.0)	[26]
c.4279G>A	p.E1427K	30	3 (2.0)	This study
c.2477T>C	p.L826P	19	2 (1.3)	[27]
c.3491G>A	p.R1164Q	24	2 (1.3)	[28]
c.3533T>C	p.L1178P	25	2 (1.3)	This study
c.281insG	p.I94MfsX7	3	1 (0.7)	This study
c.787G>T	p.A263S	7	1 (0.7)	This study
c.994delG	p.L332SfsX24	8	1 (0.7)	This study
c.1465C>T	p.R489W	12	1 (0.7)	This study
c.1987G>A	p.G663S	16	1 (0.7)	LOVD
c.3107T>C	p.F1036S	23	1 (0.7)	This study
c.3490C>A	p.R1164X	24	1 (0.7)	[7]
c.4015C>T	p.R1339C	28	1 (0.7)	[7]
c.4375G>A	p.R1459H	30	1 (0.7)	This study

Table 4.

Variant	Protein	Predicted program		
		PolyPhen2	SIFT	FATHMM
c.1256G>A*	**R419Q**	**Probably Damaging (0.994)**	**Deleterious (0.002)**	**Deleterious (-2.87)**
c.1465C>T*	**R489W**	**Probably Damaging (1)**	**Deleterious (0)**	**Deleterious (-2.62)**
c.1987G>A	**G663S**	**Probably Damaging (1)**	**Deleterious (0)**	**Deleterious (-7.27)**
c.3107T>C*	**F1036S**	**Probably Damaging (1)**	**Deleterious (0)**	**Deleterious (-4.08)**
c.3412C>T	**R1138W**	**Probably Damaging (1)**	**Deleterious (0)**	**Deleterious (-3.76)**
c.3533T>C*	**L1178P**	**Probably Damaging (1)**	**Deleterious (0)**	**Deleterious (-2.88)**
c.4015C>T	**R1339C**	**Probably Damaging (1)**	**Deleterious (0)**	**Deleterious (-3.57)**
c.4069C>T	**R1357W**	**Probably Damaging (1)**	**Deleterious (0)**	**Deleterious (-3.47)**
c.4279G>A*	**E1427K**	**Probably Damaging (1)**	**Deleterious (0.001)**	**Deleterious (-4.75)**
c.4376G>A*	**R1459H**	**Probably Damaging (1)**	**Deleterious (0)**	**Deleterious (-2.06)**
c.787G>T*	A263S	Benign (0.218)	Tolerated (0.59)	Deleterious (-3.61)
c.2477T>C	L826P	Probably Damaging (0.997)	Deleterious (0.011)	Tolerated (-0.68)
c.3491G>A	R1164Q	Possibly Damaging (0.575)	Tolerated (0.235)	Deleterious (-2.57)

* Novel missense

Table 5.

	DS (n = 54)	AS (42/50)	CVD (9/52)	HT (9/46)
Group A (n = 35)	m = 3.51 (n = 35)	88.2% (30/34)	17.6% (6/34)	20.7% (6/29)
Group B (n = 10)	m = 4.20 (n = 10)	66.7% (6/9)	33.3% (3/9)	25.0% (2/8)
Group C (n = 9)	m = 3.11 (n = 9)	85.7% (6/7)	0% (0/9)	11.1% (1/9)
p value		0.29	0.52	0.80

Table 6.

	Skin	Eye	Vascular	Cardio	Gastro
Group A (n = 35)	0.94 (n = 35)	1.94 (n = 33)	0.48 (n = 31)	0.29 (n = 34)	0.03 (n = 34)
Group B (n = 10)	1.00 (n = 10)	1.78 (n = 9)	0.25 (n = 8)	0.11 (n = 9)	0.13 (n = 8)
Group C (n = 9)	1.11 (n = 9)	2.29 (n = 7)	0.67 (n = 6)	0.13 (n = 8)	0 (n = 8)
p value	0.16	0.72	0.24	0.48	0.38

Figure 1.

Extracellular

Plasma
membrane

Intracellular

NH2

COOH

NBF1

NBF2

Figure 2.

(a) exon 1

M   WT   #51   #52   #53

469 bp

(b) exon 3

M   WT   #51   #52   #53

241 bp
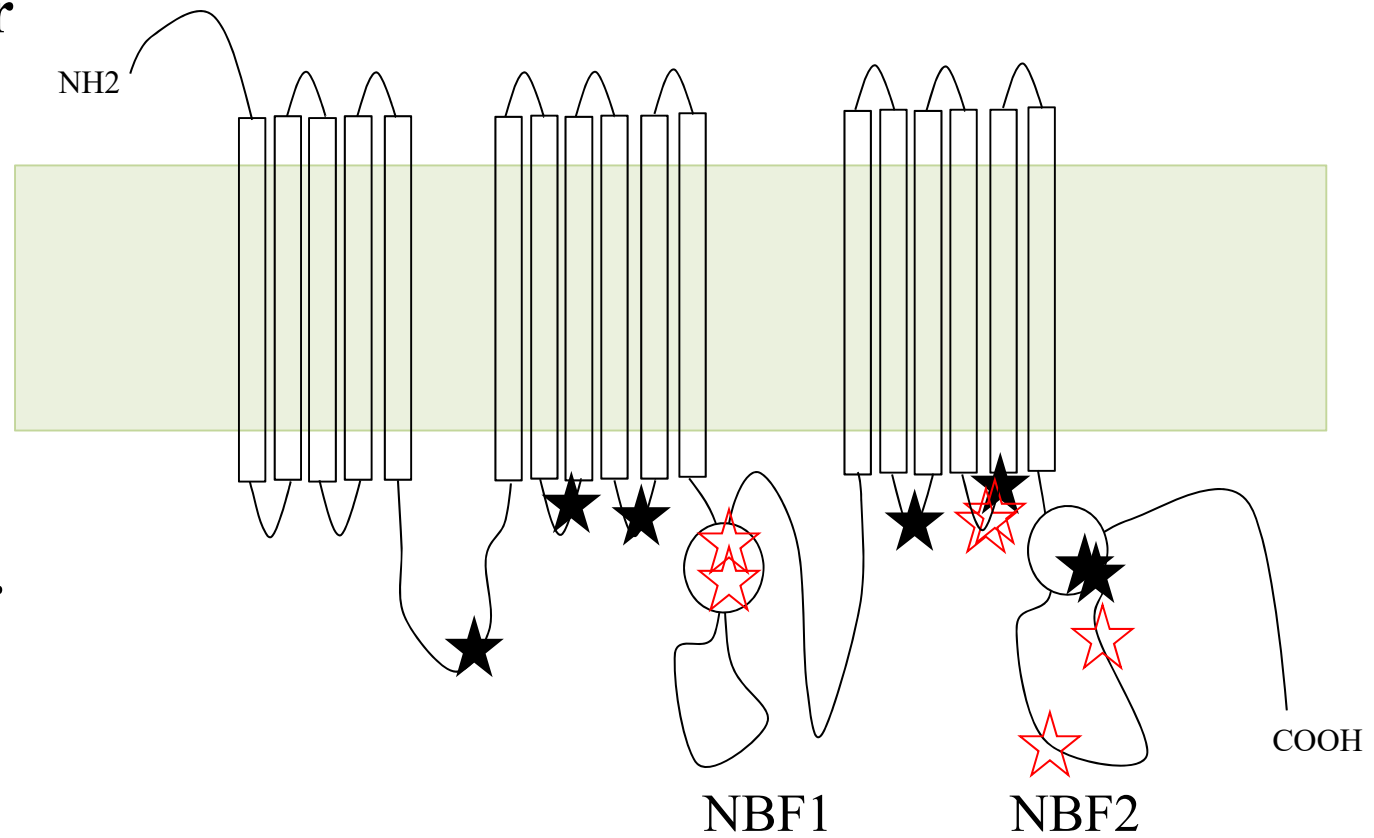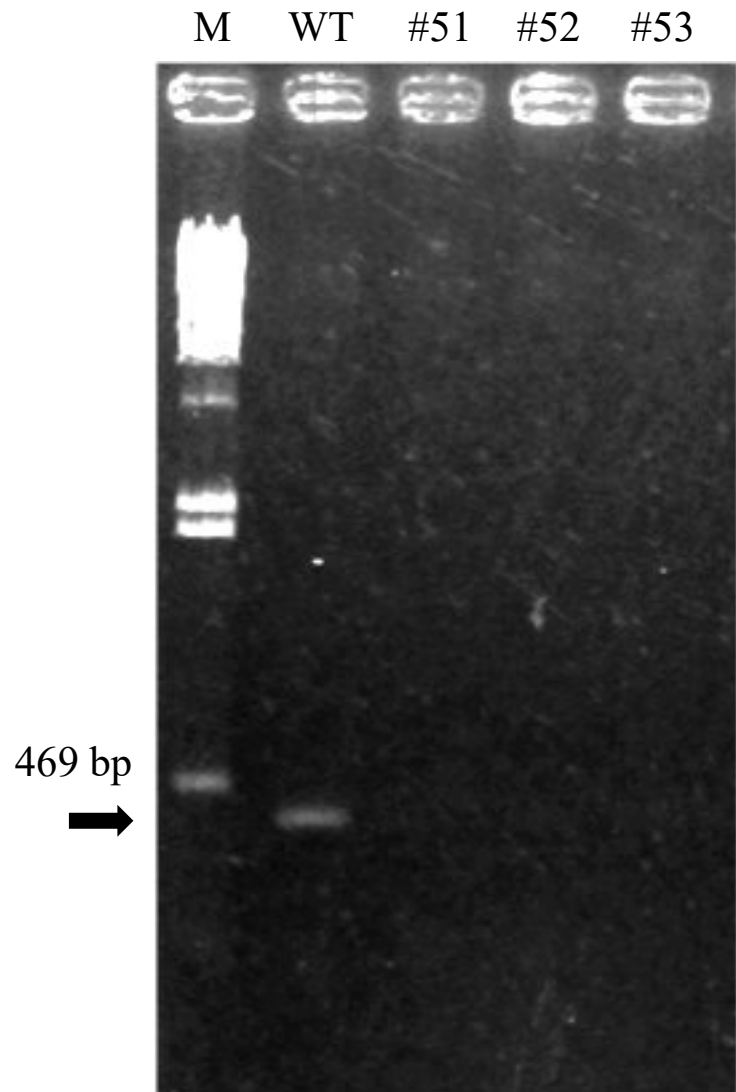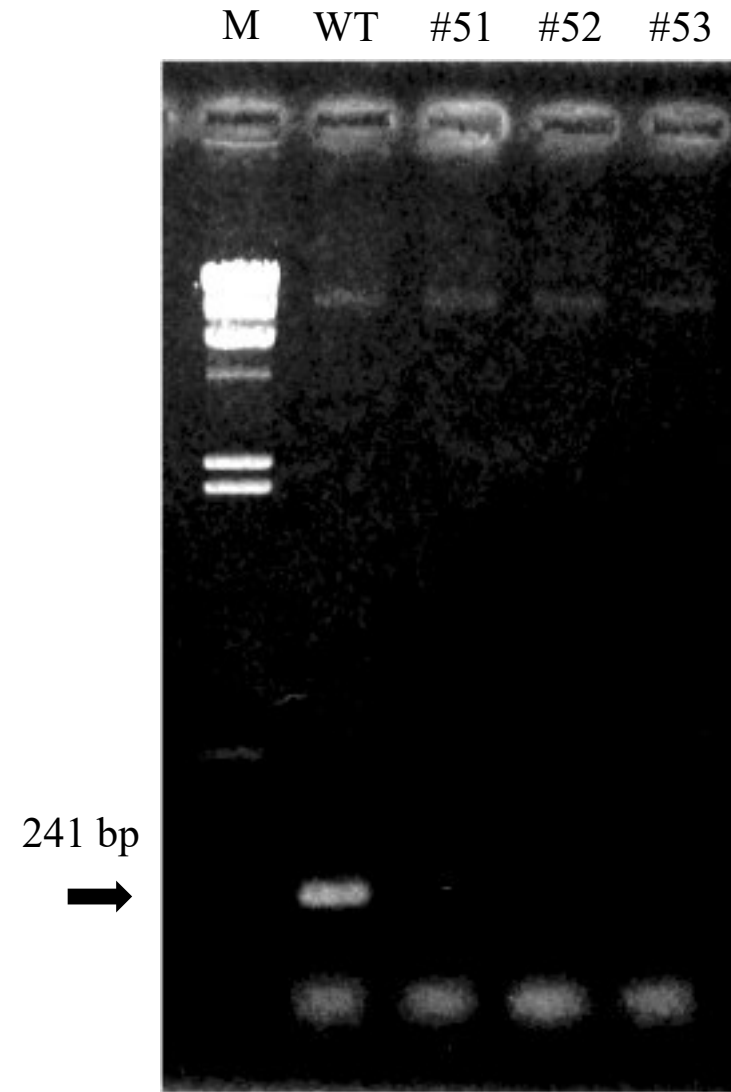
Table S1.

Organ/system	Skin			
Phenodex™ score	S0	S1	S2	S3
Symptom	No sign	Papules/bumps	Plaques of coalesced papules	Lax and redundant skin

Organ/system	Eye			
Phenodex™ score	E0	E1	E2	E3
Symptom	No sign	Peau d'orange	Angioid streaks	Bleeding and/or scarring

Organ/system	Vascular			
Phenodex™ score	V0	V1	V2	V3
Symptom	No sign	Weak or absent pulses	Intermittent claudication	Vascular surgery

Table S1.

Organ/system	Cardiac		
Phenodex™ score	C0	C1	C2
Symptom	No sign	Chest pain/angina/abnormal EKG or abnormal stress test with no symptoms	Heart attack

Organ/system	Gastrointestinal	
Phenodex™ score	G0	G1
Symptom	No sign	Bleeding (must be diagnosed as related to PXE)

Table S2.

Forward primer (5'-3' orientation)		Reverse primer (5'-3' orientation)	
E1F	TGCTGGGTCCAAAGTGTTTA	E1R	CAGCCCGAGAGATCTGCAGC
E2F	GATCCAAAAAGTTGCCTGGC	E2R	TGTCCCCTGCCTCCCCCGAA
E3/4F	TCCCAGTTGGACATGGGGCC	E3/4R	TATAAGTGTGTGCATCGTGT
E5F	CCTCTGTCTCCATTCCTTAT	E5R	AGACTGAGACCTCAAAGTGG
E6F	CACAGTTCGTCCTGTCTTCC	E6R	GGCCCTGGAGAAGCAGCTGT
E7F	GATCCTGCAGGGGTGAATGG	E7R	ATGATGAGCTTTTCTGAAGT
E8F	CCCCCAACTCCCATGATTGC	E8R	AAGGATGCCACTAAGAGACC
E9F	GAGGAGCTGCAGTCAGATTGA	E9R	GGTGACAGAGCAAGACTCCA
E10F	TTGGCCTAAGAGACTTTACTCACC	E10R	AACAAGGGTAAAACCTTTCATGTG
E11F	CAGGAAGGTCTTTGTGACCTG	E11R	ACTGTCCATTGAGAGGATAGGG
E12F	CTGAGAGGCAGACAGGTTT	E12R	AACAGGATCCAGAATGAGTG
E13F	GAGAGGACATGTGTTAGCAGGAC	E13R	GTGTTTTGCTGTCTCTCTGCC
E14F	CATCGTTTCCCATGAACTAGAAAG	E14R	GTACACCCAGGATGGTACAAAGTG
E15F	AATTTGTTCAGTGGGAGAGA	E15R	CCTTCAGGAGGTAGAGATGT
E16F	TCCTCAAATAGCTAGACAAGGACTG	E16R	CACAACTTACTTTGGTCACAGGAG
E17F	CCAAGTTCACTTTCACTCATTCTC	E17R	TGAGCTGAGCCCTTTTT
E18F	GTGTAGGTAACTCCTCCAGGAAGC	E18R	TACATAGCATTGTCACAGCAAAAG
E19F	GGCTGGTCTCGAACTTCT	E19R	TAAGAGAGCTGTCTGCTTCC
E20F	AGTGAATGCCTGAAGGATGTTC	E20R	CCTAATCATCTTGGCTAACTGGAC
E21F	AGAAGGGAAGTGTGATATCTGGTC	E21R	AGCTATGATTACATCACTGCGGTC
E22F	ACTAGCTCCCTGGGGATTGTATAG	E22R	AGACGTTTTGCACACTGTTCC
E23F	CCATCATCATGCTACTGCACTTC	E23R	AGAAAGACTGTAGTGTCCCTGTCC
E24F	CCATAGAACTCTGATTCTGCAAGG	E24R	GTGAGAACTGATAGACTGCCTGTG
E25F	AGACCCTAAAGTGGGCTTAGTTG	E25R	CTTAGCTGAGTCTGGCTCTTGTAC
E26F	GTTCTACTGAAGGAAGAGAGGGAC	E26R	TGTGACTCTGACCTATAGTGGTGG
E27F	ATTCCCATAACAACCCTGTAAAAC	E27R	GATGAGGAAGTCACCAGATG
E28F	TGGAAGGTAGACCTTTACACAATG	E28R	AAGGCTTTGAGCTGCAC
E29F	AGCTGAGGGTGGGATCT	E29R	ATAAAGGCTATCAGTAGCCCTGTG
E30F	ATCAGTTCTGCAGACCACAGAC	E30R	AGAAGTCCTGCTTTCCATGC
E31-1F	ACACATGCCAAGTGGGAAAG	E31-1R	AAAAGTACACACAGCATGGCAG
E31-2F	ACAACTGGAGAACAGAGCAT	E31-2R	AAAGTGGCCAATTATCACAG

Table S3.

Pt.	Age	Sex	Variant 1	Protein 1	Type 1	Exon	Reference	Variant 2	Protein 2	Type 2	Exon	Reference	Skin	Eye	Gastro	Vascular	Cardiac
1	41	F	2542,43 del G	fsX	SD	19	[21]	2542,43 del G	fsX	SD	19	[21]	S1	E3	G0	V0	C1
2	64	F	2542,43 del G	fsX	SD	19	[21]	2542,43 del G	fsX	SD	19	[21]	S1	E3	G0	V1	C0
3	76	M	2542,43 del G	fsX	SD	19	[21]	2542,43 del G	fsX	SD	19	[21]	S0	E3	G0	V0	C0
4	26	F	2542,43 del G	fsX	SD	19	[21]	2542,43 del G	fsX	SD	19	[21]	S1	E2	G0	V0	C0
5	65	F	2542,43 del G	fsX	SD	19	[21]	2542,43 del G	fsX	SD	19	[21]	S1	E0	G0	V0	C0
6	71	M	2542,43 del G	fsX	SD	19	[21]	c.1132 C>T	Q378X	NS	9	[25]	S1	E3	G0	V0	C0
7	27	F	2542,43 del G	fsX	SD	19	[21]	c.1132 C>T	Q378X	NS	9	[25]	S1	E2	G0	V0	C1
8	60	M	2542,43 del G	fsX	SD	19	[21]	c.1132 C>T	Q378X	NS	9	[25]	S1	E2	G0	V0	C1
9	57	F	2542,43 del G	fsX	SD	19	[21]	c.1132 C>T	Q378X	NS	9	[25]	S1	E0	G0	V0	C0
10	46	F	2542,43 del G	fsX	SD	19	[21]	c.1132 C>T	Q378X	NS	9	[25]	S1	E2	G0	V0	C0
11	63	F	2542,43 del G	fsX	SD	19	[21]	Exon2,4 deletion	Deletion	LD	2,4	This study	S1	E3	G0	V2	C0
12	72	M	2542,43 del G	fsX	SD	19	[21]	Exon2,4 deletion	Deletion	LD	2,4	This study	S1	E2	G0	V0	C1
13	26	F	2542,43 del G	fsX	SD	19	[21]	Exon2,4 deletion	Deletion	LD	2,4	This study	S0	E2	G0	V0	C0
14	70	F	2542,43 del G	fsX	SD	19	[21]	Exon2,4 deletion	Deletion	LD	2,4	This study	S1	E0	G0	-	C0
15	62	F	2542,43 del G	fsX	SD	19	[21]	c.595 C>T	Q199X	NS	5	[22]	S1	E2	G1	V0	C0
16	68	F	2542,43 del G	fsX	SD	19	[21]	c.595 C>T	Q199X	NS	5	[26]	S1	E2	G0	V0	C1
17	62	F	2542,43 del G	fsX	SD	19	[21]	994,95 del C	fsX	SD	8	This study	S1	E2	-	-	-
18	60	F	2542,43 del G	fsX	SD	19	[21]	c.1256 G>A	R419Q	MS	10	This study	S1	E2	-	-	-
19	70	F	2542,43 del G	fsX	SD	19	[21]	c.1465 C>T	R489W	MS	12	This study	S1	E0	G1	V0	C0
20	65	F	2542,43 del G	fsX	SD	19	[21]	c.4015 C>T	R1339C	MS	28	[7]	S1	E2	-	V0	C0
21	66	F	2542,43 del G	fsX	SD	19	[21]	c.4279 G>A	E1427K	MS	30	This study	S1	E2	G0	V0	C0
22	62	F	2542,43 del G	fsX	SD	19	[21]	-	-	-	-	-	S1	E3	G0	V2	C2
23	64	M	2542,43 del G	fsX	SD	19	[21]	-	-	-	-	-	S1	E2	G1	V2	C2
24	52	M	2542,43 del G	fsX	SD	19	[21]	-	-	-	-	-	S1	E2	G1	V1	C1
25	43	M	2542,43 del G	fsX	SD	19	[21]	-	-	-	-	-	S1	E2	G0	V0	C1
26	26	F	2542,43 del G	fsX	SD	19	[21]	-	-	-	-	-	S1	E2	G0	V1	C0

Table S3.

Pt.	Age	Sex	Variant 1	Protein 1	Type 1	Exon	Reference	Variant 2	Protein 2	Type 2	Exon	Reference	Skin	Eye	Gastro	Vascular	Cardiac
27	40	M	2542,43 del G	fsX	SD	19	[21]	-	-	-	-	-	S1	E2	G0	V0	C0
28	57	F	2542,43 del G	fsX	SD	19	[21]	-	-	-	-	-	S1	E2	G0	V0	C0
29	73	M	2542,43 del G	fsX	SD	19	[21]	-	-	-	-	-	S1	-	G0	V0	C1
30	38	F	c.1132 C>T	Q378X	NS	9	[25]	Exon2,4 deletion	Deletion	LD	2,4	This study	S1	E2	G0	V0	C0
31	39	F	c.1132 C>T	Q378X	NS	9	[25]	Exon2,4 deletion	Deletion	LD	2,4	This study	S1	-	G0	V0	C0
32	62	F	c.1132 C>T	Q378X	NS	9	[25]	c.1132 C>T	Q378X	NS	9	[25]	S1	E3	G0	V1	C0
33	58	F	c.1132 C>T	Q378X	NS	9	[25]	c.1132 C>T	Q378X	NS	9	[25]	S1	E2	G0	V1	C0
34	68	F	c.1132 C>T	Q378X	NS	9	[25]	c.1132 C>T	Q378X	NS	9	[25]	S1	E2	G0	V0	C0
35	22	F	c.1132 C>T	Q378X	NS	9	[25]	c.1132 C>T	Q378X	NS	9	[25]	S1	E2	G0	V0	C0
36	62	F	c.1132 C>T	Q378X	NS	9	[25]	c.595 C>T	Q199X	NS	5	[22]	S1	E0	G0	V1	C1
37	67	F	c.1132 C>T	Q378X	NS	9	[25]	c.3490 C>A	R1164X	NS	24	[7]	S1	E3	G0	V3	C2
38	62	F	c.1132 C>T	Q378X	NS	9	[25]	c.1256 G>A	R419Q	MS	10	This study	S1	E3	G0	V0	C0
39	54	F	c.1132 C>T	Q378X	NS	9	[25]	c.3107 T>C	F1036S	MS	23	This study	S1	E3	G0	V0	C0
40	47	F	c.1132 C>T	Q378X	NS	9	[25]	c.4069 C>T	R1357W	MS	29	[26]	S1	E2	G0	V0	C1
41	36	F	c.1132 C>T	Q378X	NS	9	[25]	c.4069 C>T	R1357W	MS	29	[26]	S1	E2	G0	V0	C0
42	88	F	c.1132 C>T	Q378X	NS	9	[25]	c.787 G>T	A263S	MS	7	This study	S1	-	G0	-	C0
43	48	F	c.1132 C>T	Q378X	NS	9	[25]	-	-	-	-	-	S1	E2	G0	V2	C0
44	58	F	c.1132 C>T	Q378X	NS	9	[25]	-	-	-	-	-	S1	E2	G0	V1	C0
45	66	M	c.1132 C>T	Q378X	NS	9	[25]	-	-	-	-	-	S1	E2	G0	V0	C0
46	65	M	c.1132 C>T	Q378X	NS	9	[25]	-	-	-	-	-	S1	E2	G0	-	C0
47	51	F	c.1132 C>T	Q378X	NS	9	[25]	-	-	-	-	-	S1	E0	G0	V2	C0
48	4	M	c.1132 C>T	Q378X	NS	9	[25]	-	-	-	-	-	S1	E0	G0	V0	C0
49	42	F	c.1132 C>T	Q378X	NS	9	[25]	-	-	-	-	-	S1	E0	G0	-	C0
50	75	M	c.1132 C>T	Q378X	NS	9	[25]	-	-	-	-	-	S1	E0	-	-	C0
51	62	F	Exon2,4 deletion	Deletion	LD	2,4	This study	Exon2,4 deletion	Deletion	LD	2,4	This study	S1	E3	G0	V0	C1
52	58	F	Exon2,4 deletion	Deletion	LD	2,4	This study	Exon2,4 deletion	Deletion	LD	2,4	This study	S1	E3	G0	-	C0

Table S3.

Pt.	Age	Sex	Variant 1	Protein 1	Type 1	Exon	Reference	Variant 2	Protein 2	Type 2	Exon	Reference	Skin	Eye	Gastro	Vascular	Cardiac
53	62	F	Exon2,4 deletion	Deletion	LD	2,4	This study	Exon2,4 deletion	Deletion	LD	2,4	This study	S1	E0	G0	V1	C0
54	34	F	Exon2,4 deletion	Deletion	LD	2,4	This study	c.595 C>T	Q199X	NS	5	[22]	S1	E2	G0	V1	C0
55	8	F	Exon2,4 deletion	Deletion	LD	2,4	This study	c.595 C>T	Q199X	NS	5	[22]	S1	E0	G0	-	C0
56	21	F	Exon2,4 deletion	Deletion	LD	2,4	This study	c.4279 G>A	E1427K	MS	30	This study	S1	E0	G0	V2	C0
57	64	M	c.595 C>T	Q199X	NS	5	[22]	c.595 C>T	Q199X	NS	5	[22]	S1	E2	G0	V2	C1
58	39	M	c.595 C>T	Q199X	NS	5	[22]	c.595 C>T	Q199X	NS	5	[22]	S1	E2	G0	V1	C0
59	55	M	c.595 C>T	Q199X	NS	5	[22]	281,282 insG	fsX	SI	3	This study	S1	-	G0	V1	C0
60	45	F	c.595 C>T	Q199X	NS	5	[22]	-	-	-	-	-	S1	E2	G0	V0	C2
61	53	M	3936,37 ins G	fsX	SI	28	This study	3936,37 ins G	fsX	SI	28	This study	S1	E3	G0	V0	C0
62	41	F	3936,37 ins G	fsX	SI	28	This study	3936,37 ins G	fsX	SI	28	This study	S1	E2	G0	V0	C0
63	38	F	c.1256 G>A	R419Q	MS	10	This study	c.1256 G>A	R419Q	MS	10	This study	S1	E2	G0	V0	C0
64	63	F	c.1256 G>A	R419Q	MS	10	This study	c.1256 G>A	R419Q	MS	10	This study	S1	E2	G0	V0	C0
65	31	F	c.1256 G>A	R419Q	MS	10	This study	c.4069 C>T	R1357W	MS	29	[26]	S1	E2	G0	V1	C0
66	72	M	c.1256 G>A	R419Q	MS	10	This study	c.3412 C>T	R1138W	MS	24	[4]	S1	E2	G0	-	C1
67	59	M	c.1256 G>A	R419Q	MS	10	This study	-	-	-	-	-	S1	E3	-	-	C0
68	63	M	c.3412 C>T	R1138W	MS	24	[4]	c.3412 C>T	R1138W	MS	24	[4]	S1	E3	G0	V1	-
69	67	F	c.3491 G>A	R1164Q	MS	24	[28]	c.3491 G>A	R1164Q	MS	24	[28]	S1	E3	G0	-	C0
70	60	M	c.3533 T>C	L1178P	MS	25	This study	c.3533 T>C	L1178P	MS	25	This study	S2	E2	G0	V1	C0
71	51	F	c.2477 T>C	L826P	MS	19	[27]	c.2477 T>C	L826P	MS	19	[27]	S1	-	G0	V1	C0
72	60	F	c.1987 G>A	G663S	MS	16	No reference	c.4376 G>A	R1459H	MS	30	This study	S1	-	-	-	C0
73	58	M	c.4279 G>A	E1427K	MS	30	This study	-	-	-	-	-	S1	E0	G0	V1	C2
74	56	M	-	-	-	-	-	-	-	-	-	-	S1	E2	G0	V0	C0
75	68	M	-	-	-	-	-	-	-	-	-	-	S1	E0	G0	V0	C0
76	64	M	-	-	-	-	-	-	-	-	-	-	S0	E2	G0	-	C0