

Analysis of the Frequent R1141X Mutation in the *ABCC6* Gene in Pseudoxanthoma Elasticum

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PURPOSE. To characterize the *ABCC6* R1141X nonsense mutation, which is implicated in more than 25% of a cohort of patients from The Netherlands with pseudoxanthoma elasticum (PXE).

METHODS. A combination of single-strand conformational polymorphism (SSCP), PCR, sequencing, and Southern blot analysis was used to identify mutations in the *ABCC6* gene in 62 patients. Haplotypes of 16 patients with the R1141X mutation were determined with eight polymorphic markers spanning the *ABCC6* locus. The effect of the R1141X mutation on the expression of *ABCC6* was studied in leukocytes and cultured dermal fibroblasts from affected skin in patients heterozygous or homozygous for the R1141X mutation. *ABCC6* expression was analyzed by RT-PCR and immunocytochemistry with *ABCC6*-specific monoclonal antibodies.

RESULTS. The *ABCC6* R1141X mutation was found on 19 alleles in 16 patients with PXE and occurred in heterozygous, homozygous, or compound heterozygous form. All R1141X alleles were associated with a common haplotype, covering at least three intragenic *ABCC6* markers. None of the patients or healthy control subjects had a similar *ABCC6* haplotype. Furthermore, the results showed that the expression of the normal allele in R1141X heterozygotes was predominant, whereas no detectable, or very low, *ABCC6* mRNA levels were found in R1141X homozygotes. Immunocytochemical staining of cultured dermal fibroblasts with *ABCC6*-specific monoclonal antibodies showed no evidence of the presence of a truncated protein in patients with PXE who were homozygous for R1141X.

CONCLUSIONS. A specific founder effect for the R1141X mutation exists in Dutch patients with PXE. The R1141X mutation induces instability of the aberrant mRNA. Functional haploinsufficiency or loss of function of *ABCC6* caused by mechanisms,

such as nonsense-mediated decay (NMD), may be involved in the PXE phenotype. (*Invest Ophthalmol Vis Sci.* 2003;44:1824–1829) DOI:10.1167/iov.02-0981

Pseudoxanthoma elasticum (PXE) is an autosomal inherited disorder. Patients have a spectrum of ocular abnormalities, skin lesions, and various cardiovascular complications.

Ocular signs eventually develop in most patients with PXE. Initially, eye abnormalities consist of peau d'orange or mottled hyperpigmentation of the retina. Subsequently, cometlike streaks, pinpoint white lesions of the choroid, sometimes with a hypopigmented tail in the retinal pigment epithelium, also called comets, and angioid streaks appear. Angioid streaks are cracks in the aging Bruch's membrane that most often radiate from the disc in a manner mimicking blood vessels and frequently a site of subretinal neovascular growth from the choroid. Eventually, a disciform macular degeneration develops that is frequently devastating to central vision.

Eye, skin, and cardiovascular abnormalities all appear to result from mineralization and calcification of elastic fibers in connective tissue of the affected tissues and organs, including the internal elastic lamina of Bruch's membrane.^{1,2}

We and others localized the PXE gene to chromosome 16, location p13.1^{3–5} and implicated *ABCC6* gene mutations in all genetic forms of PXE.^{6–8} The *ABCC6* gene, formerly called multidrug resistance protein 6 (*MRP6*), is a member of the adenosine triphosphate (ATP)-binding cassette (ABC) transporter superfamily C and encodes a 1503-amino-acid transmembrane protein.⁹ The protein is composed of three hydrophobic membrane-spanning domains, 17 transmembrane spanning helices and two evolutionary conserved nucleotide binding folds (NBF1 and NBF2).¹⁰ In humans, *ABCC6* is mainly expressed in liver and kidney.^{6,11,12} Using semiquantitative RT-PCR, we have found low *ABCC6* expression levels in the retina, as well as in other tissues usually affected by PXE.⁶ Recently, we have developed several monoclonal antibodies against *ABCC6* and localized the protein to the basolateral side of human hepatocytes and renal epithelial cells.¹² Evidence was obtained that *ABCC6* transports glutathione conjugates, including leukotrien-C4 (LTC4) and *N*-ethylmaleimide *S*-glutathione (NEM-GS).¹³ Loss of *ABCC6* function associated with three mutations was found to be involved in PXE.¹³ However, the functional relationship between specific substrates transported by *ABCC6* and the accumulation of abnormal elastic fibers in PXE remains to be elucidated.

At least 57 distinct *ABCC6* mutations associated with PXE have been observed in different populations. These include nonsense, missense, and putative splice site mutations, as well as deletions and an insertion.^{6,7,8,14,15} Most mutations were located toward the carboxy terminal end of the protein, and formed three clusters: in the NBF1 domain, in the 8th cytoplasmic loop between the 15th and 16th transmembrane regions, and in NBF2. The frequency of mutations in the eighth cytoplasmic loop was higher than those in the NBF1 and NBF2 domains,¹⁵ which suggests that this domain is critical for normal protein function. Although the autosomal recessive form of PXE (arPXE) is the predominant form of the disease, differ-

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ent molecular mechanisms may be involved in various types of PXE. No clear phenotype-genotype correlation has been established for the *ABCC6* gene mutations so far. The mechanism of calcification of elastic fibers in Bruch's membrane and skin is not known. Therefore, it is important to study specific *ABCC6* gene mutations in more detail at both the RNA and protein levels. Such studies may contribute to the understanding of the pathologic molecular cause underlying PXE and the clarifying of the functionally important regions of the protein.

In this study, the recurrence of the R1141X mutation in 16 patients with PXE and the level of expression of *ABCC6* mRNA with the R1141X mutation in (PXE) leukocytes and (PXE) fibroblasts were analyzed. In addition, the *ABCC6* protein in cultured dermal fibroblasts from a patient homozygous for R1141X was studied with *ABCC6*-specific monoclonal antibodies.

MATERIALS AND METHODS

Patients

PXE-affected families and sporadic cases were recruited with informed consent from the Medical Ethical Committee of the Academic Medical Center in Amsterdam. They were of Dutch descent and were primarily ascertained through the national register of genetic eye diseases at The Netherlands Ophthalmic Research Institute. Sixteen Dutch families and patients with the R1141X mutation were included in the study. Genealogical studies show that patients were not related up to four prior generations.

The diagnosis of PXE in individuals was based on the results of dermatological and ophthalmologic examinations. Most patients had obvious skin lesions. In six patients without any obvious skin lesions, skin biopsy specimens were taken from the neck, and sections were stained by the von Kossa method. In 15 patients, recent ophthalmologic examination included visual acuity measurement, slit lamp examination, ophthalmoscopy, and often fluorescein angiography. In one patient, medical records indicated the presence of angioid streaks. In six patients, cardiovascular examinations were performed that included electrocardiograms (ECGs). Control subjects for mutational analyses were spouses of (PXE and other) patients of The Netherlands Ophthalmic Research Institute and the Academic Medical Center in Amsterdam. Definition of ethnic origin of each subject was based on the country of birth in four generations. All investigations adhered to the tenets set forth by the Declaration of Helsinki.

Mutation Analysis

Genomic DNAs were prepared from patients' peripheral blood lymphocytes according to standard procedures. Primers used for polymerase chain reactions were selected from the published sequence of the human chromosome 16 BAC clone A-962B4 (GenBank Accession No. U91318; <http://www.ncbi.nlm.nih.gov/Genbank>; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD) and the TIGR database (<http://www.tigr.org>; provided in the public domain by The Institute for Genomic Research [TIGR], Rockville, MD). Fragments with a mobility shift in a single-strand conformational polymorphism (SSCP) assay were sequenced. The PCR, SSCP analysis, and cycle sequencing were essentially performed as we described previously.⁶ Potential intragenic deletions of genomic DNA were confirmed either by Southern blot analysis, familial segregation of CA repeats, or the consistent lack of amplification of the relevant exons. All patients with one mutant allele were analyzed further by hybridization on *EcoRI*-digested and *PstI*-digested genomic DNA using the amplified *ABCC6* exons from genomic DNA as probes. In addition, the *ABCC6* promoter region was sequenced up to 1 kb upstream of the first ATG. All coding sequences were analyzed in more than 100 healthy individuals, to distinguish between disease-causing mutations and polymorphic variants.

Restriction Analysis of the R1141X Mutation and Polymorphisms

After identification of the R1141X mutation and detection of other intragenic polymorphisms, optimized protocols were designed by using PCR and restriction analyses. The R1141X mutation leads to the loss of a *BstYI* restriction site, which was confirmed by a restriction fragment length polymorphism assay. The *BstYI* enzyme cleaves the wild-type sequence but not the mutant sequence. Similarly, the intragenic polymorphisms, 1896 C→A, 2490 C→G, and 3803 G→A were determined by the digestion of PCR fragments with enzyme *HpyCH4III*, *HeaIII*, and *BstNI*, respectively. The digested products were separated on 3% agarose gels. Allele frequencies of identified mutations and polymorphisms were determined both in our cohort of patients with PXE and in more than 100 control chromosomes from our healthy study participants.

Haplotype Analysis and Assessment of Founder Effect

We constructed haplotypes with eight polymorphic markers, which span ~1.5 cM around the *ABCC6* gene on chromosome 16 at location p13.1. These included the *ABCC6* flanking markers *D16S3060*, *972AAG1*, *962CA2*, and *D16S764* as well as the intragenic markers 3803 G→A, 2490 C→G, 1896 C→A, and CA(18). Markers were analyzed on 6% polyacrylamide gels or on 3% agarose gels after enzyme digestion. We constructed (phase-known) haplotypes of all informative alleles from patients with the R1141X mutation. Control haplotypes were determined on the basis of the allele segregation within individual families. Assessments of founder haplotypes were based on χ^2 analyses.¹⁶

Fibroblast Cultures

For functional studies, primary fibroblast cultures were established from dermal biopsy specimens of affected skin of patients with PXE and healthy control subjects. Cells were grown in RPMI (Life Technologies, Gaithersburg, MD), containing 10% fetal calf serum, and were used between the second and fourth passages. Total RNA was isolated from subconfluent cultures.

RT-PCR Analysis of the *ABCC6* Transcript

Total RNA was prepared from cultured dermal fibroblasts and peripheral blood leukocytes of patients with PXE and healthy control subjects, using the reagent (RNazol; Cinna/Biotech Laboratories, Houston, TX). For RT-PCR analysis, 4 μ g of total RNA was reverse-transcribed in the presence of oligo(dT)₁₂₋₁₈ and 200 U reverse transcriptase (Superscript II RT; Life Technologies). Part of the RT reaction product was used as a template in a PCR reaction with *ABCC6* cDNA-specific primers spanning exon 24.⁶ RT-PCR of β -actin⁶ served as a control. In heterozygotes, the ratio of expression between the wild-type and the mutant *ABCC6* allele in fibroblasts or in leukocytes was determined by cloning a mutation-specific RT-PCR product into a vector (pGEMTeasy; Promega, Madison, WI) followed by transformation of *Escherichia coli* strain DH5 α . The presence or absence of the R1141X mutation in individual clones was checked with digestion with *BstYI*, and, accordingly, the inserts of individual clones were assigned to be expression products from the mutant or wild-type allele.

Immunocytochemistry

Dermal fibroblasts were seeded onto sterile glass coverslips and cultured for an additional 24 hours. Cells were washed with PBS, fixed for 7 minutes in acetone at room temperature, incubated with primary rat monoclonal antibodies (1:10) for 1 hour (in the presence of 2% normal rabbit serum), washed, and further incubated with a Cy3-labeled goat anti-rat secondary antibody (1:400, Jackson ImmunoResearch, West Grove, PA). The slides were examined under a fluorescence microscope (Leica DMRB, Heidelberg, Germany). The monoclonal antibody

TABLE 1. Genotype and Clinical Features of 16 Patients with the R1141X Mutation

Pedigree	Allele 1	Allele 2	Skin	Eyes	Cardiovascular
25494	R1141X	Delex23-29	+	AS, MD	HT
26026	R1141X	Delex23-29	+, b+	D	D
26241	R1141X	Delex23-29	+, b+	PdO, AS	N
26007	R1141X	1944del22	+	RPE changes	N
26240	R1141X	Q749X	+, b+	AS	MVP
26273	R1141X	3775delT	D	AS, neo	D
26091	R1141X	R1141X	+, b+	AS	GI hemorrhage
26101	R1141X	R1141X	+	AS	TVI
26107	R1141X	R1141X	D	Neo	D
26093	R1141X	WT	+	AS	N
26123	R1141X	WT	+, b+	PdO, comet	N
24694	R1141X	WT	+	AS, MD	CI
25908	R1141X	WT	+	AS	D
26109	R1141X	WT	+	AS, neo	D
26215	R1141X	WT	+	+	D
26242	R1141X	WT	+, b+	PdO	MVP

WT, wild type; +, affected; b+, biopsy specimen obtained and histologic PXE changes determined after von Kossa staining; D, declined and/or no data available; AS, angioid streaks, MD, macula degeneration, PdO, peau d'orange, RPE involvement; neo, neovascularisation; comet, presence of comets; HT, hypertension; N, normal, MVP, mitral valve prolaps; GI, gastrointestinal; TVI, tricuspid valve insufficiency; CI, cerebral infarct.

ies for immunocytochemical staining were M₆II-7, MRPr1, and M5I-1 reactive to ABCC6, ABCC1, and ABCC5, respectively.^{11,12,17}

RESULTS

R1141X Mutation Analysis

Mutation analysis of the *ABCC6* gene resulted in the identification of the R1141X mutation, which accounted for up to 30% of all mutations detected in the *ABCC6* gene in our cohort of patients with PXE. The R1141X mutation was caused by a C→T substitution at nucleotide 3421 in the putative eighth intracellular loop. This mutation produces a stop codon at 1141 instead of an arginine residue and results in a shorter mRNA and, theoretically, in a C-terminal-truncated protein that lacks part of the transmembrane domain and the second ATP-binding domain. Previously, we found that the R1141X mutation was associated with a strong increase in the prevalence of coronary artery disease.¹⁸

In our PXE cohort, R1141X was found in 19 of 29 nondeletion alleles in DNA of 16 patients with PXE. A summary of the results is presented in Table 1. In three patients, the R1141X mutation was observed in a homozygous form; in seven, it was in heterozygous form; and in six, it occurred in combination with other mutations in the second allele in compound heterozygous form.

In three of these six compound heterozygotes (from family pedigree [P]25494, P26026, P26241), an additional intragenic 16.5-kb deletion was present. When we sequenced the fragments spanning the deletion region, we found the same break points in all three patients at nucleotide 47322 within intron 22 and nucleotide 30869 within intron 29. This seven-exon deletion is predicted to result in a polypeptide that lacks 405 amino acids.

In the three other compound heterozygotes, the combination R1141X with a del22 bp in exon 16, a 3775delT, or Q749X was identified. In family P26007, the proband showed a 22-bp deletion in exon 16, which created a premature stop codon at position 688, and which resulted in the loss of eight amino acids (Arg-Ile-Asn-Leu-Thr-Val-Pro-Glu [648-655]) of putative protein sequence. In family P26273, the proband inherited a maternal deletion of a T at cDNA

position 3775 in exon 27, which resulted in a frameshift at codon 1259 and premature termination at codon 1272. The last patient, P26240, inherited the R1141X mutation and another nonsense mutation, Q749X. The latter mutation occurred in NBF1 of the *ABCC6* protein.

Founder Effect for the R1141X Mutation

To determine whether the R1141X mutation originates from recurrent de novo mutational events or from founder effects, *ABCC6*-associated haplotypes of all patients carrying the R1141X mutation and of control subjects were constructed. Characteristics and frequency of eight markers, which spanned approximately 1.5 cM of DNA, are summarized in Table 2.

We detected 11 polymorphisms in the exonic *ABCC6* sequences. Three of these were used in this study to construct the following haplotypes: (1) 1896 C→A in exon 15 predicting an H632Q substitution, (2) 2490 C→G in exon 19 predicting an A830G substitution, and (3) 3803 G→A in exon 27 predicting a R1268Q substitution. These dimorphisms could easily be detected by PCR and restriction analysis (Fig. 1). The C→A change at nucleotide 1896 in exon 15 occurred in 24 of 32 alleles (75.0%) in the patients and 80 of 204 alleles (39.2%) in the control subjects. Statistical analysis showed a significant difference between the patients and the control subjects ($\chi^2 = 32.1$, $P < 0.001$). The second dimorphism (2490 C→G) occurred frequently in the patient group (86.2%) but rarely in the control group (14.2%). Highly significant differences were found between patients and control subjects for this dimorphism ($\chi^2 = 135.3$, $P < 0.0001$). The dimorphism (3803 G→A) revealed no differences between the patient and control groups. Both groups had 30% wild-type alleles.

The minimum common haplotype shared by all 19 alleles with the R1141X mutation was represented by the haplotype G(3803G→A)-G(2490C→G)-A(1896C→A) (Table 2). None of 10 control subjects carried this haplotype. In 17 patients, this common haplotype (G-G-A) was extended to at least one extra marker allele, allele 4 (962CA2), distal to *ABCC6*. Thirteen of the latter group shared at least one intragenic marker allele, CA(18), located close to the 5' end of the *ABCC6* gene, or even larger regions around the disease-related gene.

TABLE 2. Haplotype of the R1141X Mutation Alleles

Pedigree	Marker									
	D16S 3060	972AA AG1	962 CA2	3803 G→A	*3421* C→T	2490 C→G	1896 C→A	CA (18)	D16S 764	
25908	7	3	4	<i>G</i>	<i>T</i>	<i>G</i>	<i>A</i>	<i>1</i>	<i>3</i>	
26273	5	3	4	<i>G</i>	<i>T</i>	<i>G</i>	<i>A</i>	<i>1</i>	<i>3</i>	
25494	2	3	4	<i>G</i>	<i>T</i>	<i>G</i>	<i>A</i>	<i>1</i>	<i>3</i>	
26240	3	2	4	<i>G</i>	<i>T</i>	<i>G</i>	<i>A</i>	<i>1</i>	<i>3</i>	
24694	1	2	4	<i>G</i>	<i>T</i>	<i>G</i>	<i>A</i>	<i>1</i>	<i>3</i>	
26109		2	4	<i>G</i>	<i>T</i>	<i>G</i>	<i>A</i>	<i>1</i>	<i>3</i>	
26241	4	2	4	<i>G</i>	<i>T</i>	<i>G</i>	<i>A</i>	<i>1</i>	<i>3</i>	
26101	2	3	4	<i>G</i>	<i>T</i>	<i>G</i>	<i>A</i>	<i>1</i>	<i>2</i>	
26101	5	3	4	<i>G</i>	<i>T</i>	<i>G</i>	<i>A</i>	<i>5</i>	<i>1</i>	
26026	4	3	4	<i>G</i>	<i>T</i>	<i>G</i>	<i>A</i>	<i>1</i>	<i>1</i>	
26091	4	3	4	<i>G</i>	<i>T</i>	<i>G</i>	<i>A</i>	<i>1</i>	<i>1</i>	
26091	4	12	4	<i>G</i>	<i>T</i>	<i>G</i>	<i>A</i>	<i>1</i>	<i>2</i>	
26093	2	2	4	<i>G</i>	<i>T</i>	<i>G</i>	<i>A</i>	<i>1</i>	<i>1</i>	
26007	4	12	4	<i>G</i>	<i>T</i>	<i>G</i>	<i>A</i>	<i>1</i>	<i>2</i>	
26107	2	13	4	<i>G</i>	<i>T</i>	<i>G</i>	<i>A</i>	<i>3</i>	<i>2</i>	
26107	2	13	4	<i>G</i>	<i>T</i>	<i>G</i>	<i>A</i>	<i>3</i>	<i>3</i>	
26123	2	2	4	<i>G</i>	<i>T</i>	<i>G</i>	<i>A</i>	<i>5</i>	<i>4</i>	
26215	1	2	3	<i>G</i>	<i>T</i>	<i>G</i>	<i>A</i>	<i>4</i>	<i>3</i>	
26242	2	3	2	<i>G</i>	<i>T</i>	<i>G</i>	<i>A</i>	<i>4</i>	<i>2</i>	

Distinct alleles are indicated by number. Identical haplotypes for the R1141X mutation are shaded. Marker alleles present within the *ABCC6* gene are presented in italic. Underline shows homozygosity for the R1141X mutation in the proband(s).

*R1141X mutation.

Effect of R1141X on *ABCC6* Expression in Dermal Fibroblasts

The *ABCC6* mRNA with the R1141X mutation encodes a C-terminally truncated *ABCC6* protein that lacks part of one of the transmembrane domains and one of the ATP-binding cassette domains. To determine the effect of this mutation on the expression of the *ABCC6* gene, we analyzed dermal fibroblasts from individuals homozygous or heterozygous for R1141X and healthy control subjects. The presence of the mutations was confirmed by PCR amplification of exon 24 containing the R1141X mutation followed by restriction fragment length polymorphism analysis (Fig. 2) and direct sequencing (not shown). Next, we analyzed the amount of mRNA from alleles with the R1141X mutation. RT-PCR analyses of cultured PXE fibroblasts with the R1141X in homozygous form did not contain detectable *ABCC6* mRNA. Fibroblasts from those heterozygous for the R1141X mutation appeared to have a reduced level of *ABCC6* mRNA compared with the healthy control subjects (Fig. 3). The latter result indicates that the R1141X mutation affects the abundance of the mutant mRNA. To examine this in more detail, we determined the ratio of steady state transcript

levels between wild-type and mutated mRNA in mononuclear blood cells and fibroblasts of patients heterozygous for the R1141X mutation. As a control for the latter analysis, we also determined this ratio in blood cells from a healthy donor and from a patient with PXE heterozygous for a missense mutation R1459C in exon 30 of *ABCC6*. In fibroblasts of patients heterozygous for the R1141X mutation, no mRNA containing the mutation was found, whereas in blood cells, only 5% of the *ABCC6* mRNA was from the mutated allele (Table 3). In contrast, the relative amount of mRNA carrying the R1459C mutation (48%) was very similar to that of the wild-type mRNA (52%; Table 3), whereas the cells of a healthy donor contained only wild-type mRNA.

In parallel experiments, we determined the expression of the R1141X truncated protein in cells of patients with PXE. Cultured dermal fibroblasts of a patient with PXE homozygous for the R1141X mutation and that of a healthy control subject were analyzed by immunocytochemistry. As is shown in Figure 4, staining with a monoclonal antibody against *ABCC6* showed only staining of the control fibroblasts but not of the PXE fibroblasts. Incubation with a monoclonal antibody against *ABCC1* indicated that this protein was expressed in both normal and PXE fibroblasts at similar levels. *ABCC5* appeared not to be expressed at detectable levels by human fibroblasts, as

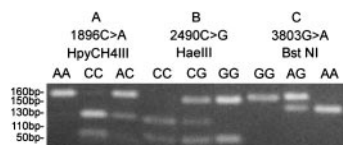


FIGURE 1. Allele-specific restriction analysis of the three dimorphisms in the *ABCC6* gene. PCR fragments were amplified using primers on exon 15, 19, and 27, followed by digestion with *HpyCH4III*, *HaeIII*, or *BstNI*, respectively. (A) DNA fragments of exon 15 were digested by *HpyCH4III*, which cuts the sequence only if a C is present at position 1896C in the sequence. (B) DNA fragments of exon 19 were digested by *HaeIII*, which cuts the sequence only if a C is present at cDNA position 2490. (C) PCR fragments of exon 27 were digested with *BstNI*, which cuts the sequence only if an A is present at position 3803. The dimorphisms and the restriction enzymes by which wild-type and variation allele were separated are depicted. The possible genotypes for each polymorphism are illustrated.

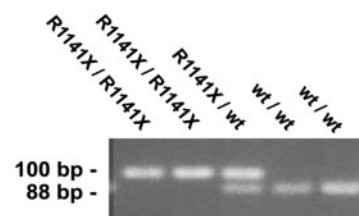


FIGURE 2. The presence of the R1141X mutation was confirmed by digestion with *BstYI* of PCR products containing exon 24. Digestion products were size fractionated on 3% agarose gel. For the wild-type allele, this results in an 88- and a 12-bp fragment. The R1141X mutant allele leads to the loss of the *BstYI* restriction site and produces a single fragment of 100 bp. The 12-bp fragment is not visible.

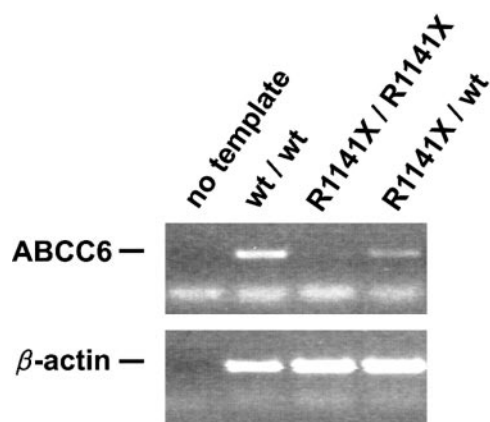


FIGURE 3. *Top*: RT-PCR analysis of *ABCC6* expression in cultured dermal fibroblasts from patients heterozygous or homozygous for the R1141X mutation. Fibroblasts from a healthy donor were also analyzed. *Bottom*: shows the control PCR for β -actin expression.

incubation with a monoclonal antibody against this protein gave no staining above background (not shown). These results suggest that the R1141X mutation in *ABCC6* does not lead to detectable amounts of truncated protein.

Phenotypes of Patients with the R1141X Mutation

A summary of clinical data available from the 16 patients with PXE with R1141X mutations is shown in Table 1. All probands had either clinically obvious PXE skin lesions or had typical PXE abnormalities detected by von Kossa staining. Angioid streaks were present in 10 patients. Six patients from the latter group had neovascularization or macular degeneration. Variable cardiovascular abnormalities were detected in six patients.

In summary, in all patients homozygous or compound heterozygous for the R1141X mutation, we observed ocular and skin abnormalities and, less frequently, cardiovascular problems. However, because the expression of the disease in these tissues is highly variable among our patients, we could not correlate a distinct phenotype with the R1141X mutation.

DISCUSSION

R1141X Mutation Analysis

We detected the R1141X mutation in homozygous, heterozygous, and compound heterozygous forms. In nine patients the R1141X mutation was present in a homozygous form or a compound heterozygous form. This is compatible with the frequently observed autosomal recessive inheritance of the disease. In seven patients, we detected R1141X in heterozy-

TABLE 3. The Expression Ratio of *ABCC6* Wild-Type and Mutated mRNA

Genotype	Tissue	WT Allele (%)	Mutant Allele (%)
WT/WT	Blood	20/20 (100)	No
R1141X/WT	Blood	38/40 (95)	2/40 (5)
R1459C/WT	Blood	52/100 (52)	48/100 (48)

The number of PXE heterozygotes carrying an *ABCC6* R1141X (R1141X/X) or R1459C (R1459C/WT) mutation and a healthy control subject with wild-type *ABCC6* (WT/WT).

Data are number of subjects/total in group, with the percentage of the total group in parentheses.

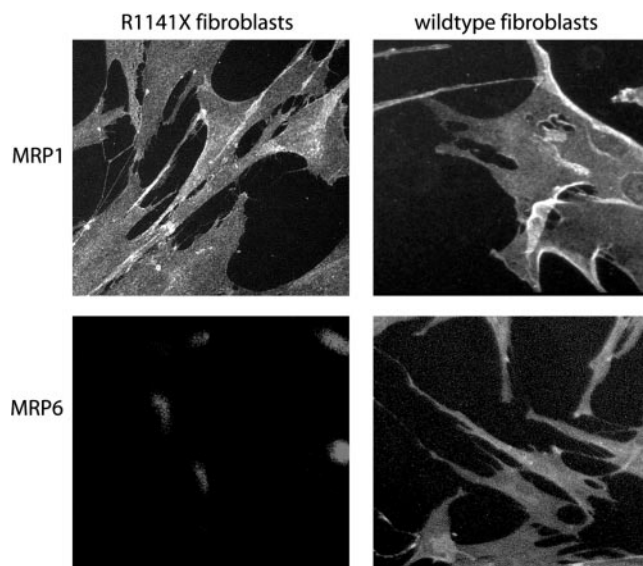


FIGURE 4. Staining with MRP1 and MRP6 monoclonal antibodies of a monolayer of dermal fibroblasts from a healthy individual and a patient with PXE heterozygous for the R1141X mutation.

gous form. These patients were either sporadic or were members of families in which autosomal recessive inheritance was the most likely segregation pattern. However, despite extensive screening, we have not yet found another mutation or deletion in the second, non-R1141X, *ABCC6* allele. Given a mutation detection frequency of approximately 50% to 55.3% (mutations per allele) in European cohorts, the most likely explanation of our results is that we still missed a substantial amount of mutations. Consequently, the pathologic molecular aspect of the R1141X mutation is most likely compatible with the frequently occurring autosomal recessive inheritance in PXE. Nonetheless, potential mild expression of the disease in carriers of the R1141X mutation warrants further investigation.

Founder Effect for the R1141X Mutation

Mutation analysis of the *ABCC6* gene in patients with PXE has yielded 57 different *ABCC6* mutations to date.¹⁵ The R1141X mutation was reported to be the most common mutation by us and others, especially in European patients.^{14,15} Recently, we also found that R1141X may be associated with a strong increase in the prevalence of coronary artery disease.¹⁸ The association between its high frequency and the geographical distribution could reflect a founder effect from a common ancestor. To test this hypothesis, we analyzed the R1141X mutation in more detail in this study.

The majority of our R1141X mutant alleles (17/19) shared a common haplotype spanning at least one *ABCC6* flanking marker. Our results and statistical analysis suggested that a founder effect exists in the Dutch PXE group. In only two patients did partial aberrations of the consensus haplotype occur. These could be due to (ancient) recombination events including CA(18), 972AAAG1, and D16S764.

Identification of founder effects in the local population, as presented in this study, can greatly simplify genetic analysis of the disease, because, initially, the founder mutation can be rapidly screened in all patients. Associated clinical studies may provide further accurate information for genetic counseling and prenatal diagnosis.

Predominant Expression of the Normal *ABCC6* Allele in Patients with PXE Heterozygous for the R1141X Mutation

For several mammalian mRNAs, it has been shown that a nonsense mutation or a frameshift mutation that generates a nonsense codon may greatly influence the abundance of these transcripts. A specific mechanism called nonsense-mediated mRNA decay (NMD) accelerates decay of transcripts coding for truncated proteins and thus minimizes potential metabolic damage.^{19,20} We found no detectable *ABCC6* mRNA in patients with PXE who were homozygous for the R1141X mutation. Consistent with this observation, no *ABCC6* protein was detected in cultured dermal fibroblasts of a patient homozygous for R1141X. Using a more quantitative approach, we found that in cultured dermal fibroblasts of a R1141X heterozygote, only transcripts from the wild-type allele were detected. In mononuclear blood cells of a R1141X heterozygote the mutated transcript was detected, but the abundance was reduced to 5% of total *ABCC6* mRNA.

Our results suggest that the R1141X mutation induces instability of the aberrant *ABCC6* mRNA, which leads to a reduced abundance of the corresponding transcript due to alterations in RNA processing by NMD. The latter mechanism may in part be an explanation of the obvious variability in the expression of the disease. The possibility that NMD contributes to a particular phenotype has also been suggested for other genes, such as fibrillin 1 in Marfan syndrome²¹ and β -globin in beta zero-thalassemia.^{22,23} So far, because of the small size of the families, we have not established a clear correlation between the level of *ABCC6* mRNA and the patient's phenotype. However, it is reasonable to assume that dosage-dependent severity caused by the presence of NMD of mRNA may be involved in PXE. Complete loss of *ABCC6* function causes PXE in homozygotes or compound heterozygotes, whereas partial loss of *ABCC6* function in heterozygotes may result in a variable phenotype ranging from no signs at all to the complete PXE phenotype.

Features of the Phenotype

The clinical variability in PXE was demonstrated previously^{2,24} and also occurred in our R1141X patient cohort. In this study, we could not firmly predict the phenotype from the genotype, or vice versa. The correlation between genotype and phenotype may be obscured by several factors. The small size of our cohort limited the evaluation of the genotype and phenotype correlation. In addition, additional unknown environmental, metabolic, or genetic determinants may modify the phenotype. In future studies, we have to investigate the PXE phenotype in a thorough prospective way to obtain any significant clues for genotype-phenotype relationships.

CONCLUSION

In summary, this study presents evidence that the frequent occurrence of the *ABCC6* R1141X mutation in Dutch patients with PXE was due to a founder effect. The PXE phenotype of the R1141X mutation is most likely due to a complete loss of function or functional haploinsufficiency of the *ABCC6* gene. No clear correlation between the R1141X genotype and phenotype could be established in the cohort studied. Further analysis of additional PXE families with this mutation should help to increase our understanding of the function of the *ABCC6* gene and the molecular pathology underlying PXE.

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