Novel *ABCC6* Mutations in Pseudoxanthoma Elasticum

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Pseudoxanthoma elasticum (PXE) is a heritable connective tissue disorder caused by mutations in an ABC (ATP-Binding Cassette) transporter gene (*ABCC6*), which manifests with cutaneous, ophthalmologic, and cardiovascular findings. We studied a cohort of 19 families with PXE, and identified 16 different mutations, nine of which were novel variants. The mutation detection rate was about 77%. We found that arginine codon 518 was, with the previously described R1141X and EX23_29del, a recurrently mutated amino acid (11.5% of the mutations detected for each variant R518Q and R518X). No clear delineation of genotype/phenotype correlation was identified, and marked intra-familial variability of the disease was seen in one family. One family with pseudodominant inheritance displayed three distinct *ABCC6* mutations, providing further evidence for the probable exclusive recessive transmission of PXE. These data contribute to the expanding database of *ABCC6* mutations, to the description of phenotypic variability, and inheritance in PXE, and should be helpful for genetic counselling.

Key words: pseudoxanthoma elasticum/MRP6/ABCCG/pseudodominant inheritance/mutation
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Pseudoxanthoma elasticum (PXE) is an inherited disorder involving mainly three organ systems (cutaneous, ocular, and cardiovascular) with highly variable clinical expression. Recessive inheritance is common, but dominant inheritance has been suspected (Pope, 1975; Neldner, 1988; Bergen *et al*, 2000). Skin lesions manifest as yellowish papules that tend to coalesce into plaques of inelastic, redundant, and sagging skin. These lesions are primarily seen on the neck, axilla, antecubital fossa, groin, and periumbilical area. They are often the first clinical manifestations leading to diagnosis. The diagnosis is confirmed by the demonstration of fragmented calcified elastic fibers using the von Kossa stain in lesional skin. Eye lesions mainly consist of the so-called angioid streaks, resulting from pathological fractures in the elastin-rich sheath of the retina (Bruch's membrane). Neovascularization, as a complication of angioid streaks, may cause recurrent hemorrhages and subsequent visual impairment. Cardiovascular involvement manifests as early arteriosclerosis, occlusive vascular changes, and rupture of blood vessels (particularly within the gastrointestinal tract) with considerable morbidity (Neldner, 1988; Lebwohl *et al*, 1993).

Recently, several groups identified *ABCC6*, an ATP-binding cassette transporter gene encoding a multidrug resistance protein (MRP6), as the defective gene in PXE (Bergen *et al*, 2000; Le Saux *et al*, 2000; Ringpfeil *et al*, 2000; Struk *et al*, 2000). To date, 57 mutations have been identified in patients with PXE (Bergen *et al*, 2000; Le Saux *et al*, 2000, 2001; Ringpfeil *et al*, 2000, 2001; Struk *et al*, 2000; Cai *et al*, 2001; Meloni *et al*, 2001; Uitto *et al*, 2001; Hu *et al*, 2003). *ABCC6* is mostly expressed in human liver and kidneys at both mRNA and protein levels (Kool *et al*, 1999; Scheffer *et al*, 2002), and to a lesser extent in human tissues affected with PXE (Bergen *et al*, 2000). *In vitro* studies (Belinsky *et al*, 2002; Cai *et al*, 2002; Ilias *et al*, 2002) have shown that mutations in *ABCC6* lead to impaired transport of glutathione conjugates, but the physiological metabolite(s) actively transported by MRP6 are not yet identified.

Abbreviations: ABC, ATP-binding cassette; PXE, pseudoxanthoma elasticum

Results and Discussion

Mutation detection Sixteen pathogenic mutations were identified in the 19 families studied, and are summarized in Table I. The overall detection rate of the disease-causing sequence variants was 77%. At least one mutation was found in 16 individuals (84%). Nine new mutations were identified. Among these, five are missense mutations (R391G, A766D, D1238H, L1335P, E1400K), one is a nonsense mutation (W1223X), one is a small in-frame deletion of 33 bp (1088–1120del), and two are predicted to impair splicing (V74del, IVS25-3C$>$A).

All new mutations but three (R391G identified in family 15, D1238H identified in family 11, and E1400K identified in families 1 and 3) fulfil the criteria described for defining nucleotide sequence variants as disease-causing (Cotton and Scriver, 1998). Although R391, D1238, and E1400 are not fully conserved among the 12 known human ABCC proteins (ClustalW program), these mutations cosegregate with the PXE phenotype in the four families and were not found in a panel of 200 alleles from unaffected control individuals. R391 was considered as a conserved amino

Table I. Phenotype and genotype of the patients studied

Patient	Origin	Sex	Age (y)	Amino acid variation	Nucleotide variation	Exon	Phenotype		
							C	O	CV
1	France/Greece	F	26	R1141X	3421C>T	24	1	0	0
				E1400K	**4198G>A**	29			
2	France	F	31	**V74del**	**220-222del**	3	1	1	0
				V74del	**220-222del**	3			
3-1	Morocco	F	20	**E1400K**	**4198G>A**	29	1	0	0
				E1400K	**4198G>A**	29			
3-2		M	18	**E1400K**	**4198G>A**	29	1	0	2
				E1400K	**4198G>A**	29			
4	France	F	36	A999_S1403del	EX23_29del	23–29	1	1	0
				Splicing alteration	**IVS25-3C>A**				
5	France	F	44	?/?			2	0	0
6	France	M	15	R1141X	3421C>T	24	2	0	1
				R1141X	3421C>T	24			
7	Morocco	F	26	R518Q	1553G>A	12	1	1	1
				R518Q	1553G>A	12			
8	Turkey	F	21	**A766D**	**2297C>A**	18	1	0	0
				A766D	**2297C>A**	18			
9	France	F	41	R518Q	1553G>A	12	1	0	0
				T1130M	3389C>T	24			
10	France	F	30	R518X	1552C>T	12	1	0	0
				R518X	1552C>T	12			
11-1	Algeria	F	75	NA			1	0	0
				T1130M	3389C>T	24			
11-2		M	39	**D1238H**	**3712G>C**	26	1	0	0
11-3		F	36	**Q363_R373del**	**1088-1120del**	9	2	0	0
				D1238H	**3712G>C**	26			
12	France	M	58	?/?			0	2	0
13	France	F	50	**L1335P**/?	**4004T>C**	28	1	0	0
14	France	M	51	?/?			2	2	1
15	France	F	50	**R391G**	**1171A>G**	9	1	0	0
				A999_S1403del	EX23_29del	23–29			
16	France	M	42	R1138Q	3413G>A	24	1	0	0
				R1138Q	3413G>A	24			
17	France	F	35	R518X/?	1552C>T	12	1	0	0
18	France	F	31	R1141X	3421C>T	24	1	0	0
				W1223X	**3668G>A**	26			
19-1	France	M	18	R1164X/?	3490C>T	24	1	0	0
19-2		M	15	R1164X/?	3490C>T	24	1	0	0

The nine novel mutations are in bold.
C (cutaneous manifestations): 0 —clinically absent but present on biopsy examination, 1 —moderate skin lesions (neck and folds), 2 —extended skin lesions.
O (ophthalmologic manifestations): 0 —absence (absence of angioid streaks or absence of neovascularization),
1 —angioid streaks with neovascularization or visual impairment, 2 —central blindness.
CV (cardiovascular manifestations): 0 —absence or pulse abolition, 1 —symptomatic (arteriosclerosis), 2 —hemorrhagic or ischemic complication.
NA: not available.

acid among 11 ABCC proteins (ABCC1 to ABCC11), but as non-conserved when ABCC12 was added. ABCC12, however, encodes an unusual ABC protein without the second ATP-binding domain and contains only four membrane-spanning regions in the carboxyl half of the protein (Bera *et al*, 2002). Therefore, R391G was assumed to be disease-causing. D1238H and E1400K drastically change the charge of residues located close to (D1238H) or in (E1400K) the second nucleotide binding domain (NBD2) of the molecule, where mutational analysis in ABC transporters has often been shown to lead to impaired function (Kerb *et al*, 2001). For these reasons, D1238H and E1400K were assumed to be pathogenic mutations.

This study is the largest PXE study of families of French origin (15 families). The two known recurrent mutations (R1141X and EX23_29del) account respectively for 13% and 7% of the mutations detected in the French families included in our study, and were absent from the families originating from Algeria, Morocco, and Turkey. In our cohort, we found that arginine codon 518 is a new recurrent mutated amino acid, corresponding to 23% of the mutations detected in the family studied (11.5% for R518X and 11.5% for R518Q).

Nine of the mutations that we identified are novel. The nonsense mutation (W1223X), and the mutations predicted to impair exon 3 and exon 26 splicing (V74del, IVS25-3C>A) predict the production of a truncated protein, provided that the mutated mRNA is stable. The 33 bp deletion (1088–1120del) leads to the deletion of 11 amino acids located in transmembrane and intracellular domains. All the five novel missense mutations are located within intracellular domains of the molecule, three in the nucleotide binding domains (A766D in NBD1, L1335P and E1400K in NBD2) and two in a cytoplasmic loop (R391G, D1238H).

Two of the mutations (D1238H in family 11 and IVS25-3C>A in family 4), located in exon 26 and its flanking intronic sequences, were not detected by dHPLC, and were identified only after direct sequencing of all 31 exons of *ABCC6*. The lack of detection of these mutants by dHPLC could be due to the observed complex melting curve of the corresponding amplimer. The detection rate in our study is 0.72 when only screening by dHPLC is used, and 0.77 when screening by dHPLC and systematic direct sequencing are combined. This detection rate is higher than in previous reports. Possible reasons for the lack of detection in some patients could be due to exonic deletions (i.e., deletion of exon 15 (Le Saux *et al*, 2001)), splice site mutations distant from the coding sequence (i.e., mutation 1811 + 1.6 kb in the *ABCC7/CFTR* gene (Reboul *et al*, 2002)), or mutation in the regulatory sequences of the gene.

Haplotype analysis We performed haplotype analysis to identify a possible founder origin of mutations found in our study. The results are summarized in Table II. The recurrent mutated allele R1141X shares a common haplotype identical-by-descent in the French families (families 1, 6, and 18), which is consistent with the same geographic origin of these patients. R1141X is the most frequent mutation found in the European population, and haplotype analysis of additional European patients would be helpful to establish whether this mutant allele is inherited from a common ancestor. Haplotype analysis of the two French families (families 4 and 15) sharing the recurrent mutation EX 23_29del reveals different alleles, identical-by-state. This mutation is mainly identified in the North-American population in which an identical-by-descent mutated allele could not be excluded. Haplotype analysis in families homozygous for a mutation shows common haplotypes, consistent with the known consanguinity (families 2, 3, 7, and 8), or suggestive of unknown consanguinity (families 6, 10, and 16). Haplotypes identified in these families are different from the one associated with the same mutations in family 9 (R518Q), family 17 (R518X), and family 1 (E1400K), suggesting that they do not share a common founder with these families.

Genotype–phenotype correlation The clinical features of the patients are summarized in Table I together with the mutations identified. Regarding the high degree of allelic heterogeneity and the small size of the cohort, it was difficult to make correlations between the phenotype and the nature or the position of the mutations. In our study, homozygosity or compound heterozygosity for mutations leading to a premature stop codon (families 2, 4, 6, 10, and 18) was not significantly associated with a more severe phenotype. Among the missense mutations we identified, E1400K was associated with a high intra-familial phenotypic variation in family 3, suggestive of the contribution of other factors to the phenotype severity. Specifically, in this family the sister has typical skin lesions without cardiovascular or ocular involvement, whereas the brother has typical skin lesions associated with severe cardiovascular disease manifestations (multiple ischemic cerebral strokes during his infancy, renal and hepatic diffuse arteritis, internal carotid and iliac artery stenosis, and an episode of gastrointestinal hemorrhage). No other cause of cardiovascular disease was identified in this patient; in particular, the search for hemoglobinopathy, protein C and protein S deficiencies was negative. The two sibs had no major differences in nutritional, lifestyle, environmental factors, or medical history, suggesting the implication of the genetic background in the genotype variability.

Mode of inheritance PXE was first described as a sporadic disorder, but both autosomal recessive and autosomal dominant inheritances have been observed (Pope, 1975; Neldner, 1988; Bergen *et al*, 2000). No family with PXE transmitted through three generations has been published however, and both dominant and recessive forms of PXE have been linked to the same chromosomal region 16p13.1 (Struk *et al*, 1997). Since the implication of *ABCC6* mutations in PXE, no specific dominant mutation has been described. One family (family 11) in our cohort was first suggestive of dominant inheritance (Fig 1). Surprisingly, the molecular analysis found three different mutations in the two sibs: one was a compound heterozygote for T1130M and D1238H, while the other was a compound heterozygote for a 33 bp deletion in exon 9 (1088–1120del) and D1238H. The mother who was affected with PXE declined genetic analysis. The father was deceased and his status for PXE was unknown. On the basis of our molecular results however, each sib has probably inherited one of the two

Table II. Haplotypes of the mutant alleles

Mutations	Family	Origin	Known consanguinity	Haplotype										
				IVS13-86 G>T	1841 T>C	IVS14-92 delG	IVS14-90 G>T	IVS14-57 G>A	1890 C>G	1896 C>A	IVS30-76A>G	IVS30-30G>A	D16B 9621	D16B 9622
R1141X	**1**	**France**	**No**	+	+	+	−	−	+	+	−	+	**322**	**151**
	6-allele a	**France**	**No**	+	+	+	−	−	+	+	−	+	**322**	**151**
	6-allele b			+	+	+	−	−	+	+	−	+	**322**	**151**
	18	**France**	**No**	+	+	+	−	−	+	+	−	+	**322**	**151**
EX23_29del	4	France	No	+	+	+	−	−	+	+	?	?	322 or 330	165
	15	France	No	+	+	+	−	−	+	+	−	−	346	165
V74del	**2-allele a**	**France**	**Yes**	−	−	−	−	−	−	−	−	−	**322**	**165**
	2-allele b			−	−	−	−	−	−	−	−	−	**322**	**165**
R518Q	**7-allele a**	**Morocco**	**Yes**	−	−	−	+	−	−	−	−	+	**322**	**169**
	7-allele b			−	−	−	+	−	−	−	−	+	**322**	**169**
	9	France	No	?	+	?	−	−	?	?	?	+	**322**	**169**
R518X	**10-allele a**	**France**	**No**	−	−	−	−	+	−	−	−	+	**330**	**161**
	10-allele b			−	−	−	−	+	−	−	−	+	**330**	**161**
	17	France	No	−	?	?	−	−	?	?	−	−	**322**	**161**
A766D	**8-allele a**	**Turkey**	**Yes**	+	+	+	−	−	−	−	+	+	**322**	**165**
	8-allele b			+	+	+	−	−	−	−	+	+	**322**	**165**
T1130M	9	France	No	?	+	?	−	−	?	?	?	+	322	169
	11	Algeria	No	+	+	+	−	−	+	+	?	+	322	161 or 165
R1138Q	**16-allele a**	**France**	**No**	−	−	−	−	+	−	−	−	+	**322**	**161**
	16-allele b			−	−	−	−	+	−	−	−	+	**322**	**161**
E1400K	1	Greece	No	+	+	+	−	−	+	+	−	+	348	151
	3-allele a	**Morocco**	**Yes**	+	+	+	−	−	+	+	−	+	**342**	**153**
	3-allele b			+	+	+	−	−	+	+	−	+	**342**	**153**

The mutant alleles sharing the same haplotype are in bold.
When the patients are homozygous for a mutation, the two alleles are denoted by a and b.
+: presence of the polymorphism; −: absence of the polymorphism; ?: uninformative.

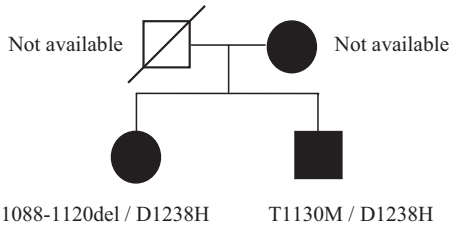

Figure 1
Family 11 with pseudodominant inheritance. The identification of three distinct mutations in the two affected sibs showed recessive inheritance. 1088–1120del and T1130M are likely to be inherited from the affected mother, while D1238H is probably inherited from the father. None of the parents' DNA was available for the study.

putative maternal mutated alleles (T1130M or 1088–1120del). We suggest that the mutated allele D1238H shared by both affected offspring has been inherited from the father, leading to recessive inheritance. Thus, this family would correspond to a case of pseudodominance. Another case of pseudodominant PXE has previously been proved by molecular analysis of *ABCC6* in a consanguineous family (Ringpfeil *et al*, 2000). *ABCC6* mutations have been reported in three families with apparently dominant PXE. In two families, the recurrent recessive mutation R1141X was identified in affected patients (Bergen *et al*, 2000). In a third family, the three affected sibs were heterozygous for the maternal inherited R1495C mutation. Autosomal recessive inheritance could not be excluded in this family, however, as the three patients inherited the same paternal allele (Hu *et al*, 2003). 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 (Sherer *et al*, 2001). Taken together, these data support a unique recessive mode of inheritance in PXE.

In summary, we have identified nine novel mutations in patients with PXE, adding to the *ABCC6* mutation database. No clear delineation of genotype/phenotype correlation was identified and we observed a marked intra-familial phenotypical variation in one family. Our study and previous publications further support an exclusively autosomal recessive mode of inheritance for PXE. This information should help clinicians for genetic counselling.

Materials and Methods

Patients A cohort consisting of 19 families with PXE was studied. The diagnosis was based on dermatological examination in combination with ophthalmological and cardiovascular evaluation. In each patient, the diagnosis was confirmed by histopathology examination of a skin biopsy combined with special stains for elastin (Verhoeff–van Gieson) and/or for calcium (von Kossa). The majority of the families (15 of 19) originated from France. In three families, two or more individuals were affected. Consanguinity was known in only four families (families 2, 3, 7, and 8) (Table I).

Mutation detection After informed consent for inclusion in the study, DNA was isolated by standard procedures from the peripheral blood of patients affected with PXE, from their unaffected relatives, and from clinically healthy unrelated individuals. Intron-derived primers specific for PCR amplification of *ABCC6* exons 1–9 (which do not amplify the two truncated pseudogenes *ABCC6*φ1 and *ABCC6*φ2) were synthesized using sequences previously reported (Pulkkinen *et al*, 2001). Intron-derived primers for PCR amplification of *ABCC6* exons 10–31 were synthesized using sequence information from BAC clone CIT987SK-A-962B4 containing ABCC6 (GenBank accession number U91318). Primers are available upon request to the authors. Intron/exon boundaries were deduced from information available from the Institute for Genome Research database and by comparison with the *ABCC6* cDNA sequence (GenBank accession number NM_001171). PCR products were analyzed by denaturing high-performance liquid chromatography (dHPLC), followed by automated sequencing of the PCR products displaying an abnormal elution profile. EX23_29del was screened by PCR using a previously described set of nested primers (Le Saux *et al*, 2001).

All 31 exons of *ABCC6* were sequenced in patients who had only one or no mutant alleles identified after mutation screening by dHPLC.

Haplotype analysis Haplotype analysis was performed for mutation identified in at least two families, using two microsatellite markers D16B9621 and D16B9622 closely linked to *ABCC6* (Cai *et al*, 2000) and nine intragenic single-nucleotide polymorphisms (1841T > C, 1890C > G, 1896C > A, IVS13-86G > T, IVS14-92delG, IVS14-90G > T, IVS14-57G > A, IVS30-76A > G, IVS30-30G > A).

Clinical investigation was carried out in Orléans and Toulouse (France), and mutation identification was performed in Toulouse (France).

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