ORIGINAL RESEARCH ARTICLE | Genetics inMedicine

Mutation spectrum in the *ABCC6* gene and genotype–phenotype correlations in a French cohort with pseudoxanthoma elasticum

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Purpose: Pseudoxanthoma elasticum (PXE) is an autosomal recessive disorder caused by variants in the *ABCC6* gene. Ectopic mineralization of connective tissues leads to skin, eye, and cardiovascular manifestations with considerable phenotypic variability of unknown cause. We aimed to identify genotype–phenotype correlations in PXE.

Methods: A molecular analysis was performed on 458 French PXE probands clinically evaluated using the Phenodex score (PS). Variant topographic analysis and genotype–phenotype correlation analysis were performed according to the number and type of identified variants.

Results: Complete molecular analysis of 306 cases allowed the identification of 538 mutational events (88% detection rate) with 142 distinct variants, of which 66 were novel. Missense variant distribution was specific to some regions and residues of *ABCC6*. For the 220 cases with a complete PS, there was a higher prevalence of eye features in Caucasian patients ($P = 0.03$) and more severe eye and vascular phenotype in patients with loss-of-function variants ($P = 0.02$ and 0.05, respectively). Nephrolithiases and strokes, absent from the PS, were prevalent features of the disorder (11 and 10%, respectively).

Conclusion: We propose an updated PS including renal and neurological features and adaptation of follow-up according to the genetic and ethnic status of PXE-affected patients.

Genet Med advance online publication 19 January 2017

Key Words: *ABCC6*; genotype–phenotype correlation; Pseudoxanthoma elasticum; Phenodex score

INTRODUCTION

Pseudoxanthoma elasticum (PXE; OMIM 264800) is a rare, autosomal recessive disorder characterized by ectopic mineralization and fragmentation of the elastic fibers of select connective tissues. The estimated prevalence lies between 1/25,000 and 1/50,000 (ref. 1). According to this prevalence, the theoretical frequency of heterozygous subjects in the population lies between 1/111 and 1/80.

The skin, eye, and cardiovascular system are primarily affected, with considerable interfamilial and intrafamilial variability. Skin abnormalities begin with yellowish papules in the flexural areas that progressively coalesce into plaques, which rarely affect nonflexural sites. Skin lesions are the most prevalent sign of PXE; they are the key features for diagnosis (97–98% of cases).[2,3] The most common PXE ocular signs are *peau d'orange* and angioid streaks (AS) in the retina, which are present in 89–96% of cases.[2] AS can be responsible for subretinal choroidal neovascularization, hemorrhage, and subsequent loss of central vision. Almost all patients present a combination of AS and skin lesions.[2,4]

Cardiovascular symptoms are loss of peripheral pulses, intermittent ischemic claudication, renovascular hypertension, angina, and, rarely, myocardial infarction. Cerebral ischemic involvement and gastrointestinal bleeding can also occur. These manifestations are the consequence of accelerated arteriosclerosis with mineralization of the internal elastic lamina of medium arteries. The cardiovascular system is less frequently affected than the skin and eyes (7 to 30% of patients have intermittent claudication); symptoms commonly appear in the third decade of life.[2,5]

The clinical diagnosis is commonly made using the consensus conference criteria focused on skin and eye manifestations,[6]

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Submitted 22 September 2016; accepted 22 November 2016; advance online publication 19 January 2017. doi:10.1038/gim.2016.213

with sensitivity and specificity close to 100%.[4] The severity of the disease can be evaluated with a scoring system called the Phenodex score (PS), which was proposed at the consensus conference in 2007 (**Supplementary Table S1** online).[3]

PXE is caused mainly by loss-of-function variants in the *ABCC6* gene[7] encoding an ATP-binding cassette (ABC) protein, which functions as an efflux transporter. Its specific substrate remains unknown, although ABCC6 was recently demonstrated to be a major regulator of plasmatic inorganic pyrophosphate, a strong ectopic mineralization inhibitor with low plasmatic inorganic pyrophosphate plasmatic levels associated with PXE.[8] Up to 300 causative variants have been identified in *ABCC6* and gathered in a dedicated database (CLINVAR: https://www.ncbi.nlm.nih.gov/clinvar/?term=ABCC6). These include missense, nonsense, and splice-site variants and deletions and insertions. Although most of them are private, two recurrent variants of high frequency have been identified: a nonsense variant in exon 24, c.3421C>T, p.Arg1141Ter, with a 25% prevalence in cases of various ethnic backgrounds,[3] and *Alu1*-mediated deletion of exons 23 to 29 (del23-29), which is the most common variant in Americans of European descent, with a prevalence of 28%.[9]

In previous case series, attempts were made to identify specific variant distributions along the protein as well as genotype–phenotype correlations.[3,10–12] Schulz et al.[12] related the severity of the disorder to complete loss of the protein and established a significant correlation between the PXE phenotype and variants in *ABCC6*. No specific variant clustering could be found in the ABCC6 protein.

Here, we present the results of phenotypic and molecular characterization of *ABCC6* in a series of 306 French PXE probands. In 220 cases, the phenotype based on the PS was well characterized, allowing genotype–phenotype correlation analysis and providing significant results.

MATERIALS AND METHODS

Study population

Unrelated PXE probands from diverse backgrounds were examined by dermatologists or geneticists across France from 1 January 2000 to 30 November 2015, and were referred to the Genetics Department of the Georges Pompidou European Hospital (Paris, France) for molecular analysis. The clinical diagnosis was based on the consensus criteria.[6] A detailed questionnaire (**Supplementary Figure S1** online) was drafted by the principal investigator and completed at the time of molecular request. It collected epidemiological and medical information including age and ethnicity (as declared by the patient), family history of PXE, and clinical findings of skin, eye, vascular, cardiac, and gastrointestinal symptoms corresponding to the PS (**Supplementary Table S1** online) and was completed using neurological and renal information. The PS was adapted in this study by adding three clinical findings: stroke or transient ischemic attack (V3-attributed), which may occur in 15% of PXE cases[13]; peripheral artery disease revealed by vascular imaging without any clinical sign (V1-attributed); and mitral valve insufficiency (C1-attributed). Age at diagnosis was approximated by the age of the molecular analysis request. The PS was calculated for each case by the primary investigator.

Written informed consent for the genetic study was obtained, and genetic testing was performed in accordance with French legislation regarding genetics diagnostics tests (French bioethics law 2004-800). The study was approved by the Ethics Committee of the Hôpitaux Universitaires Paris Ouest, Paris, France.

Genetic analysis

The Qiamp DNA Blood Midi kit (Qiagen, Hilden, Germany) was used according to the manufacturer's instructions to extract genomic DNA from leukocyte pellets. *ABCC6* exons and the flanking intronic sequences were amplified by PCR with specific primers to avoid amplification of *ABCC6* pseudogenes Ψ1 and Ψ2 (ref. 14), sequenced using BigDye Terminator kit v3.1 cycle sequencing kits, and run on an ABI Prism 3730XL DNA Analyzer (Life Technologies, Foster City, CA). DNA variants were identified using Sequencher software. Sanger sequencing was completed by a multiplex PCR targeting the recurrent deletion including exons 23 to 29 (ref. 15).

ABCC6 multiplex ligation-dependent probe amplification (MLPA) was performed to detect large rearrangements in a subset of cases with single heterozygous point variants or suspected hemizygosity based on sequencing results. The SALSA reagent set P092B kit (MRC-Holland, Amsterdam, The Netherlands) was used according to the manufacturer's recommendations (http://www.mlpa.com). Fragments were detected using an ABI 3730XL Genetic Analyzer (Life Technologies, Foster City, CA) with ROX500 (PerkinElmer Applied Biosystems, Foster City, CA) as an internal size standard. Genemapper (PerkinElmer Applied Biosystems, Foster City, CA) and Coffalyzer (MRC-Holland, Amsterdam, The Netherlands) software were used to analyze the MLPA fragments.

Array comparative genomic hybridization (array-CGH) was performed to confirm cases with large deletions with the Illumina HumanCytoSNP-12 v2.1 BeadChip array, Illumina GenomeStudio v2011.1, and Illumina cnvPartition v3.1.6 software (Illumina, San Diego, CA).

Unreported variants were interpreted with in silico prediction tools (SIFT: http://www.Blocks.fhcrc.org/sift/SIFT.html, PolyPhen-2: http://genetics.bwh.harvard.edu/pph/ and MutationTaster: http://www.mutationtaster.org/documentation.html), and their frequencies in the general population were determined using the Exome Aggregation Consortium (ExAC) database (http://exac.broadinstitute.org/gene/ENSG00000091262).

Variant-distribution analysis

Variant distribution was analyzed as follows: (i) distribution of recurrent variants according to ethnic background; (ii) distribution in intracellular (IC), extracellular (EC), and transmembrane (TM) domains compared with the general population in the ExAC database; (iii) distribution in specific domains called

nucleotide-binding fold 1 (NBF1), nucleotide-binding fold 2 (NBF2), and the eighth intracellular loop (CL8) as the ratio of mutated residues over all residues in each domain; (iv) frequency of mutated arginine residues as a ratio to all arginine residues in *ABCC6* and the corresponding frequency of C>T and G>A transitions in CpG dinucleotides.

Epidemiological, genotype–phenotype correlations, and statistical analyses

Cases with incomplete clinical or molecular data, no *ABCC6* variant, or proven a posteriori differential diagnosis were excluded from all subsequent analyses (**Figure 1**).

Ages were classified in 7 groups with 10-year intervals, with the first group comprising those younger than age 20 years and the last group those older than 70 years. Ethnicity was taken into consideration for the two ethnic groups of the cohort: Caucasian and North African individuals. The severity of the phenotype was evaluated by comparing, in each organ category (skin, eye, vascular, and cardiac), the number of cases within each class of severity (0, 1, 2, ±3). Cases were classified into two groups according to the number of variants (1 or 2 variants).

First, groups were studied according to age, sex, and ethnicity. Second, the severity was studied according to the same three parameters. When a correlation was found with the number of variants, only cases with two variants were included in further correlation analyses to exclude a bias of the variant number. Third, cases with two variants were grouped by the predicted effect of the genotype on the protein: (i) cases harboring two variants leading to presumably residual conservation of the transporter function, including missense, in-frame deletions and duplication, and variants leading to the lack of an in-frame exon (group M for missense); (ii) cases with a mixed genotype combining one missense variant and one loss-of-function variant (group LM); (iii) cases harboring a complete loss-of-function with two variants among nonsense, frameshift, out-of-frame deletions, splicing frameshift, and gene deletions (group L for loss-of-function). The phenotype severity was compared between the three groups, M, LM, and L, and between the two more distant groups, M and L.

All qualitative variables were compared using chi-square or Fisher's exact tests (when count was insufficient in the contingency table). Age distributions and global PS were reported as medians and compared using ANOVA tests. $P \leq 0.05$ was considered significant. Only significant results are discussed in this report.

RESULTS

Epidemiological and clinical characteristics of index cases

From 1 January 2000 to 30 November 2015, 458 probands were addressed for *ABCC6* analysis; 306 PXE probands with positive consensus criteria had a complete molecular analysis. Of them, 220 cases with complete PS and the presence of one or two variants could be included in the correlation analysis (**Figure 1**).

Cases were predominantly women (n = 193/306, 63.1%) and Caucasians (n = 243/306, 79.4%) with a median age of 43.5 (range 0–83). Ten percent (n = 24/239) of PXE cases had neurovascular manifestations (transient ischemic attack, stroke, or neurological sequelae of ischemia) occurring at very variable ages (median age 51.5, range 31–73). A prevalence of 10.9% (n = 26/239) of renal lithiases was also found in our cohort.

Variants

A total of 538 mutational events were identified in 612 disease alleles, corresponding to a variant detection rate of 87.7%; 142 *ABCC6* distinct variants were identified (**Figure 2a**, **Supplementary Table S2** online). These included missense variants (42.8%), nonsense (28.8%) variants, and frameshift (5.0%) variants that produce premature stop codon, splice-site variants (7.2%), large deletions (12.6%), gene deletions (2.0%), large duplications (0.4%), and small in-frame deletions (1.1%). Most variants were unique or were identified in a limited number of cases.

Eleven variants were found to be recurrent (**Supplementary Table S2** online); the common variants p.Arg1141Ter (107/538 = 19.9%) and del23-29 (60/538 = 11.2%) were the most frequent in Caucasians ($P = 8.4 \times 10^{-7}$ and 0.0035). Less common recurrent variants (p.Arg518Gln, p.Glu1400Lys, and p.Arg1314Trp) were more prevalent in cases that originated from North Africa ($P = 3.9 \times 10^{-10}$, 7.5×10^{-5} and 4.7×10^{-5}, **Figure 2b**). The rarest recurrent variants p.Gln378Ter, p.Arg391Gly, p.Thr1130Met, c.3736-1G>A, c.2787 + 1G>T, and p.Arg518Ter were not specific to any of these ethnic backgrounds. Arginine codon 518 was a recurrent mutated amino acid in our cohort: the two variants p.Arg518Gln and p.Arg518Ter corresponded to 9.9% (53/538) of the detected variants. The level of recurrence seemed different from other series for a few variants (**Figure 2b**).[3]

MLPA revealed 11 gene deletions in 11 cases, of which 5 were confirmed by array-CGH: a recurrent deletion of 1.25 Mb containing 13 genes including *ABCC1* and *MYH11* was found in 4 cases and a larger deletion of 2.6 Mb also including *XYLT1*, which was suggested as a modifier gene in PXE,[16] was identified in the last case.

Sixty-six variants were novel (**Supplementary Table S2** online) and submitted to the public database dedicated to it (https://www.ncbi.nlm.nih.gov/clinvar/?term=ABCC6).

Genotypes

Among the 306 fully genotyped cases, 6.5% (n = 20) had no identifiable variant, 11.4% (n = 35) had 1 variant, 81.7% (n = 250) had 2 variants, and 0.3% (n = 1) had 3 variants. In cases with 2 variants, almost 25% were homozygous (n = 56/250, 22.4%), of which 41.1% were from North Africa.

An unexpected apparent mode of inheritance was also observed in a few cases. Molecular analysis confirmed recessive inheritance in two pedigrees showing pseudodominant transmission (**Supplementary Figure S2a,b** online). In these families, three variants were identified: the affected parent harboring two pathogenic variants in trans and the other parent, who was heterozygous for a third variant that was transmitted to the offspring.

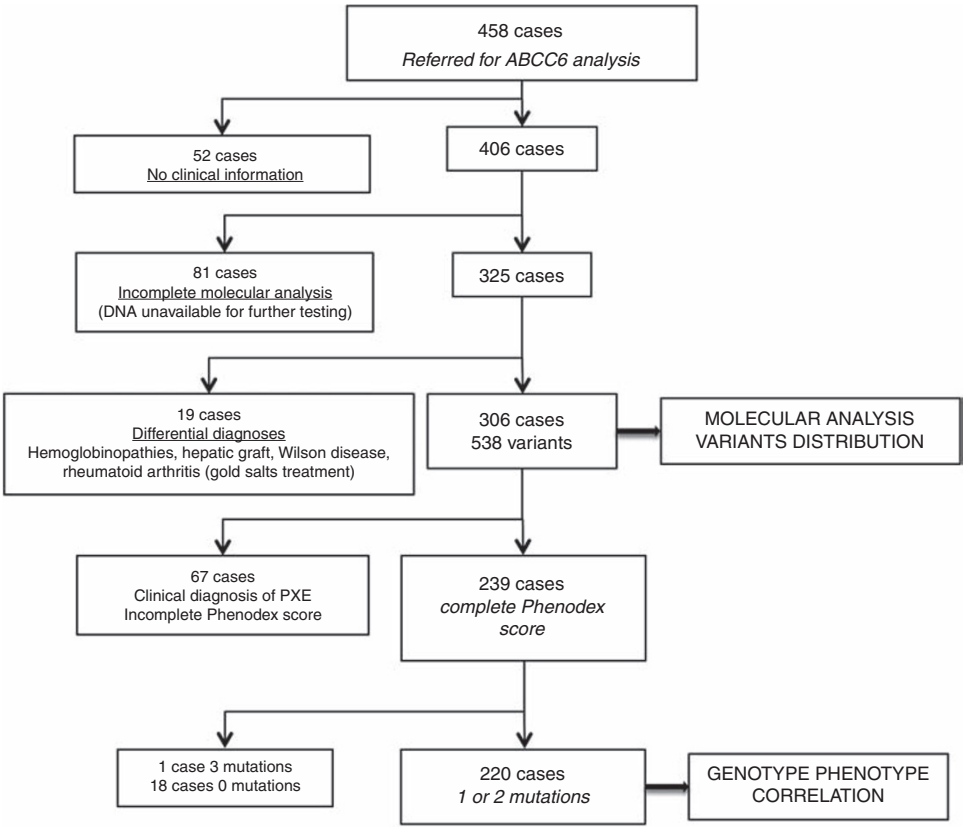

Figure 1 Flowchart of the studied cohort. Molecular data for 306 cases were analyzed, and 220 cases with a complete Phenodex score were included in the genotype–phenotype correlation analysis.

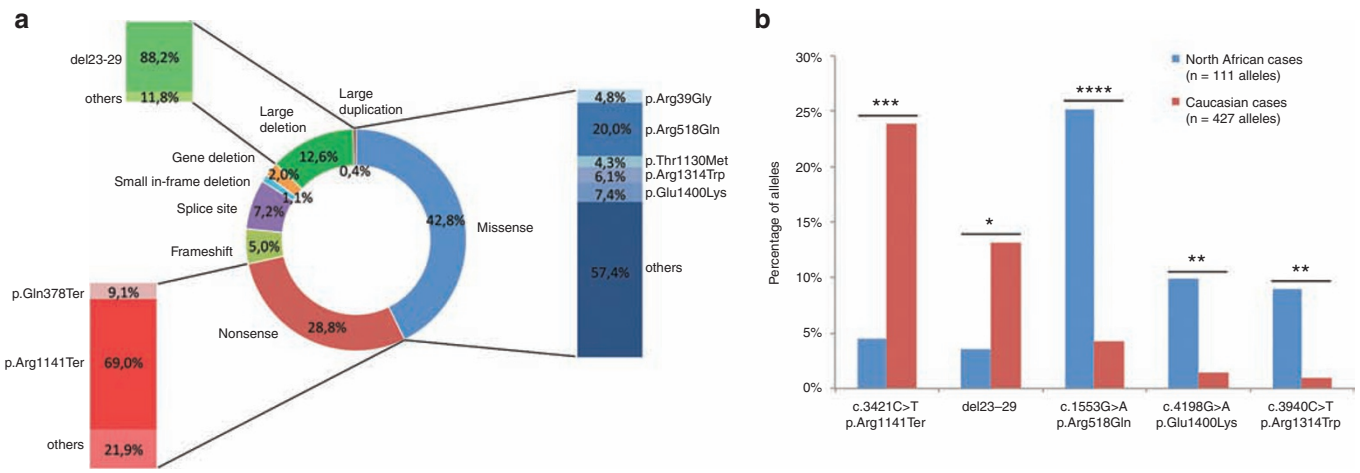

Figure 2 Frequency of *ABCC6* variants. (**a**) Frequency of types of variants in the 306 PXE cases. Missense variants were further clustered into the five most frequently identified alterations (p.Arg391Gly, p.Arg518Gln, p.Thr1130Met, p.Arg1314Trp and p.Glu1400Lys) and all the private missense variants (corresponding to "others"). Nonsense variants were also further divided into the two most frequent alterations (p.Arg1141Ter and p.Gln378Ter) and the other, private nonsense variants. Large deletions were further divided into the recurrent del23-29 and the other large deletions. (**b**) Ethnic-specific frequency of *ABCC6* recurrent variants. $*P < 0.01$, $**P < 0.0001$, $***P < 10^{-6}$, $****P < 10^{-9}$.

One case had a de novo deletion encompassing *ABCC6*, *ABCC1*, and *MYH11* in one allele, the other allele harbouring a hemizygous deletion of 33 bp in exon 9 inherited from the father (**Supplementary Figure S2c** online).

Distribution of the missense variants in the ABCC6 protein

Most missense variants were within IC domains ($n = 65$, 87%; **Figure 3**). The remaining 10 missense variants occurred in the TM ($n = 7$) and the EC ($n = 3$) domains. The distribution of

missense variants in IC versus EC plus TM domains was significantly different from population-based ExAC database variants (cohort distribution/EXAC distribution in IC: n = 65/422 vs. EC+TM: n = 10/153; P = 0.01; http://exac.broadinstitute.org/gene/ENSG00000091262), demonstrating a bias toward intracellular topography of pathogenic variants.

In IC domains, two subdomains showed significant enrichment in variant distribution regarding their relative size (**Figure 3**): 9 variants were found in the CL8 (P = 0.005) and 20 variants were found in the NBF1 (P = 0.017). The NBF2 was not significantly enriched in pathogenic variants (n = 15, P = 0.52).

Arginine residues were found to be the preferred sites of missense substitutions ($P = 6.0 \times 10^{-23}$): 37% (n = 28/75) were located at an arginine residue (n=21, 6% of the coding sequence of *ABCC6*). These substitutions were mainly C>T and G>A transitions in CpG sites (n = 24/28). These latter types of transition were found in 34% (n = 33/95) of substitutions in the coding sequence, suggesting a mechanism of deamination of methylated cytosines.

Phenotypic analysis

A phenotypic analysis was performed for the 220 cases with complete PS (**Figure 1**, **Table 1**). Skin lesions were the most prominent manifestation, regardless of the number of detected variants, and were present in more than 90% of cases with two variants (**Supplementary Table S3** online). When no variant

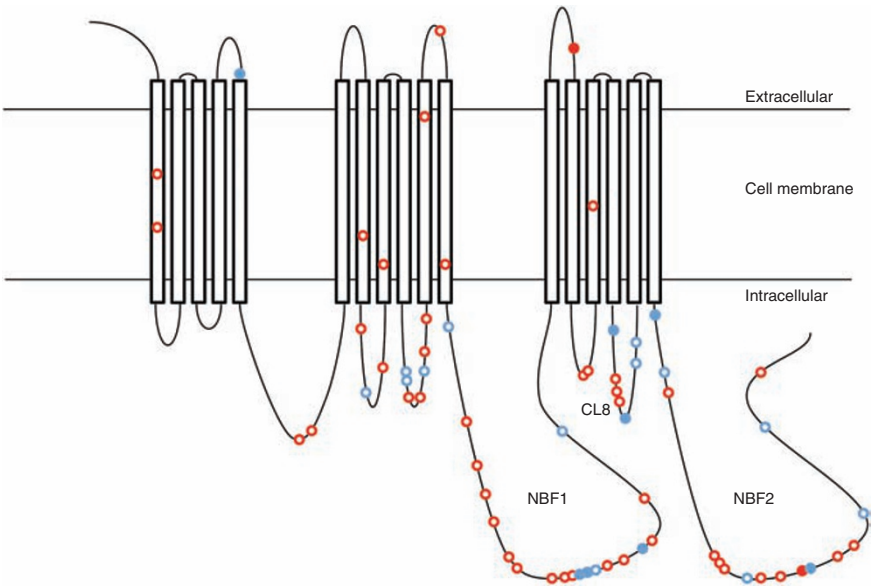

Figure 3 Schematic representation of the ABCC6 protein and the position of identified missense variants. Circles indicate mutated positions (blue: arginine residues; red: nonarginine residues; empty circles: 1 missense variant per amino acid position; full circles: ≥2 missense variants occurring at the same amino acid position). CL8: eighth intracellular loop; NBF1 and NBF2: nucleotide binding fold-1 and fold-2 domains, respectively.

Table 1 Main features and complications according to the Phenodex score and to the genotype of *ABCC6* gene in cases with two variants

	Total (n = 194)	M (n = 52)	LM (n = 79)	L (n = 63)	P (L versus M versus LM)
Sex (F/M)	123 (63.4%)/71 (36.6%)	37 (71.2%)/15 (28.8%)	50 (63.3%)/29 (36.7%)	36 (57.1%)/27 (42.9%)	NS[a]
Ethnicity (C/NA)	150 (77.3%)/44 (22.7%)	22 (42.3%)/30 (57.7%)	73 (92.4%)/6 (7.6%)	55 (87.3%)/8 (12.7%)	3.3×10^{-7} [a]
Age at genetic diagnosis	39 (8–75)	37.5 (10–75)	39 (8–75)	43 (8–73)	0.39[c]
Consanguinity	18 (9.3%)	13 (25.0%)	1 (1.3%)	4 (6.3%)	2.1×10^{-5} [b]
Skin lesions	180 (92.8%)	48 (92.3%)	71 (89.9%)	61 (96.8%)	NS[a]
Eye lesions	172 (88.6%)	46 (88.5%)	66 (83.5%)	60 (95.2%)	0.02[b]
Vascular lesions	84 (43.3%)	17 (32.7%)	40 (50.6%)	27 (42.9%)	0.05[b]
Cardiac lesions	37 (19.1%)	7 (13.5%)	17 (21.5%)	13 (20.6%)	NS[a]
Nephrolithiasis	25 (12.9%)	6 (11.5%)	11 (13.9%)	8 (12.7%)	NS[a]
Strokes	17 (8.8%)	3 (5.8%)	7 (8.7%)	7 (11.1%)	NS[b]
Gastrointestinal lesions	16 (8.2%)	5 (9.6%)	9 (11.4%)	2 (3.2%)	NS[b]
Global Phenodex score	4.5 (1–10)	4 (1–8)	5 (1–10)	5 (1–10)	NS[c]

Quantitative variables are given as medians (extremes). Group M: cases with two missense variants; group LM: cases with one missense and one loss-of-function variants; group L: cases with two loss-of-function variants.

C, Caucasian background; F, female; M, male; NA, North African background; NS, not significant.

[a]Chi-square test. [b]Fisher exact test. [c]Analysis of variance (ANOVA) test.

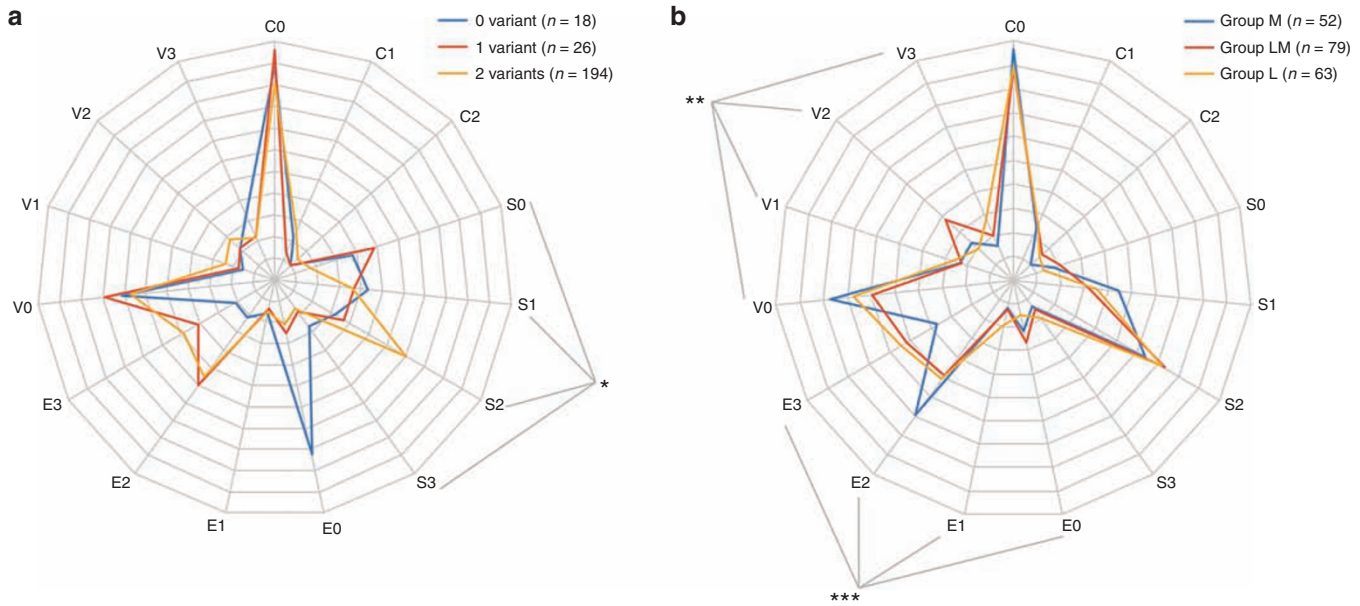

Figure 4 Radar plots for the severity of the phenotype. (**a**) Severity according to the number of identified variants. (**b**) Severity according to the type of variants in cases with two variants. *$P < 0.0001$, **$P = 0.05$, ***$P < 0.05$.

was identified, skin, eye, vascular, and cardiac manifestations were, respectively, present in almost 75%, 25%, 33%, and 10% of cases (**Supplementary Table S3** online). The most recurrent profile in this group corresponded to a score of S1E0G0V0C0, for which the skin lesions contrasted with the lack of eye complications (**Figure 4a**). In the group with one variant, eye manifestations reached more than 80% (**Supplementary Table S3** online). In this group, two phenotypic profiles predominated: S0E3G0V0C0 (15%, corresponding to an isolated eye phenotype with AS and bleeding) and S1E2G0V0C0 (15%, corresponding to mild skin lesions associated with AS; **Figure 4a**). The latter group, with two variants, had a greater involvement of cardiac complications (19%, **Supplementary Table S3** online) and gastrointestinal bleeding (8%). Most cases with two variants had a score of S2E2G0V0C0 (15%, corresponding to coalescent skin papules associated with AS; **Figure 4a**).

Genotype–phenotype correlations

Correlation analyses were performed in three steps. First, age, sex, ethnic origin, and the severity of the phenotype were tested for their association with the number of detected variants. Age and severity of the phenotype were associated with the number of variants ($P = 0.008$): cases with two variants had a younger age at diagnosis ($P = 0.046$; median age = 39 in cases with two variants and 49 in cases with one variant) and a more severe skin phenotype ($P = 9.6 \times 10^{-5}$).

Second, we analyzed the severity of the phenotype in relation to sex, ethnic origin, and age. Age at diagnosis did not seem to affect the severity of PXE, but sex was found to influence the skin and cardiac manifestations (**Supplementary Table S4a** online): skin lesions were more severe in females than in males ($P = 0.009$), with a predominance of coalescent plaques (score S2). Conversely, males were more severely affected by cardiovascular complications than females ($P = 0.007$), who usually presented with angina or abnormal electrocardiogram results (score C1). Regarding ethnic background, the eye phenotype was less severe in North Africans than in Caucasians (**Supplementary Table S4b** online), who had predominant AS and bleeding ($P = 0.03$). No difference was observed regarding other symptoms.

Third, the severity of cases with two variants was compared with regard to their genotype: 27% had compound missense variants (group M), 41% had a mixed genotype (group LM), and 32% had a complete loss-of-function (group L). Severity of the eye and vascular phenotype increased with the level of loss-of-function ($P = 0.02$ and 0.05; **Table 1**, **Figure 4b**): cases with complete loss-of-function have an increased risk of developing retinal bleeding and severe claudication leading to vascular surgery.

All types of clinical manifestations were analyzed for correlations, and vascular and cardiac complications were positively correlated ($P = 0.009$ for cases with one or two variants and $P = 0.02$ for cases with two variants).

DISCUSSION

Detection rate

The detection rate of 87.7% was roughly similar to the mean detection rate, which we evaluated to be 76% (based on the merged data of six studies[3,10,11,13,17,18]). This incomplete detection of causal variants for PXE has multiple possible explanations. First, the common strategy applied in diagnostic laboratories uses MLPA, a turnkey solution that lacks coverage for a significant part of *ABCC6* coding sequence. Completing this approach by systematic array-CGH might help to improve this point.

However, phenotypic profiles and age at diagnosis differ significantly in cases with one versus two variants (**Figure 4a** and

Supplementary Table S3 online). It is noteworthy that in cases with one variant in which familial recurrence is present, pedigrees are consistent with recessive inheritance but not dominant inheritance (data not shown). These combined observations suggest that, at least in some cases, a hypomorphic, noncoding variant that is not targeted by the current genetic analyses might be the second unidentified variant. These could correspond to single-nucleotide variants or copy-number variants in the regulatory regions, conferring a quantitative effect at the transcript and protein level. This hypothesis has been recently raised in a study showing that the copy number of *ABCC6* pseudogenes had an impact on the phenotype.[19] However, these studies need to be repeated in larger cohorts to take these data into account.

Inherited disorders related to PXE, like GACI, related to *ENPP1* deficiency, or PXE-like, related to *GGCX* deficiency, could also explain incomplete *ABCC6* genotypes.[20]

Variants and genotypes

Variant and genotype data were relatively similar to previously published data; the two most common variants p.Arg1141Ter and del23-29, the less common variants affecting Arginine codon 518, and complete gene deletions were similar to previously reported frequencies.[3,21] The frequency of a few variants seemed different from that of other series (**Figure 2b**).[3] Distinguishing the ethnic background of cases allowed the identification of specific frequencies in Caucasian and North African cases, which were previously unreported; p.Arg518Gln, p.Glu1400Lys, and p.Arg1314Trp were found to be overrepresented in North African cases.

Several unusual types of transmission were observed. Recessive inheritance was observed in two families in whom the trait segregated as pseudodominant. This result, which is in agreement with that of previous series,[22] confirms that PXE is a true recessive disorder. This is consistent with previously discussed data concerning the incomplete detection rate, for which another causal variant is to be considered in cases with one detected *ABCC6* variant.

We also identified one de novo variant. This mechanism has been reported only rarely,[7] and de novo variant prevalence seems to be very low.

Topography of the variants

Previous studies pointed to plausible clusters of variants in three intracellular domains of ABCC6, CL8, NBF1, and NBF2 (refs. 3,10,11). CL8 was suggested to be involved in the ABCC6 substrate recognition[23] with little experimental proof. By contrast, the critical role of NBFs for active ATP-dependent transport across the cell membrane has long been studied,[24] with NBF1 showing higher ATPase activity than NBF2.[25–27] Our analysis confirmed that two of these three subdomains—CL8 and NBF1—were true clusters of pathogenic variants in PXE. Conversely, the NBF2 was not significantly enriched in PXE variants.[10,17] These results confirm and highlight the major roles for NBF1, as suggested by Ostuni and Procko,[25,27] and for CL8, whose role has not been fully elucidated.

Arginine residues were found to be clear hotspots of variants, as previously suggested. Their contribution to the ABCC6 structure and function should be studied in order to understand their role in various proteic domains interactions, as previously shown for the ARA motif (Ala-Arg-Ala), which harbors variants associated with disease in several ABC transporters.[28]

Genotype–phenotype correlations

PXE is characterized by marked clinical intrafamilial heterogeneity[10,29] and significant age-dependent severity, particularly for eye and cardiovascular manifestations. Numerous modifying factors have been proposed to explain the phenotypic variability, such as modifier genes, including *ENPP1* (refs. 30,31), environmental factors such as magnesium intake,[32] and transcriptional activator sequences in the *ABCC6* gene promoter.[17] All these factors could explain the failure of previous studies to establish genotype–phenotype correlations focused on the coding sequence of *ABCC6* by increasing the background noise and decreasing the power of the analyses. In this context, the phenotypic analysis of a large French cohort using the PS provided significant results.

First, we demonstrate that the number of identified variants mostly has an impact on the age at diagnosis and little impact on the severity of the disorder, apart from skin manifestations.

Second, this study revealed that partial or complete *ABCC6* loss of function predisposes to vascular and eye complications. This was suggested in previous studies in which generalized involvement (high global PS) or significantly younger age of onset were observed in cases harboring nonsense variants.[10,12] However, these studies were based on small cohorts and little or no information was provided concerning the study design and statistical analyses.

Surprisingly, we found a correlation between the severity of the symptoms and ethnicity, because Caucasians had more severe eye complications than North Africans. This was unexpected because susceptibility to retinal diseases in general does not seem to be more frequent in individuals with a Caucasian background, and the vascular phenotype that was similar in Caucasians and North Africans could not explain this result. However, the enrichment of group M by North African cases ($P = 3.3 \times 10^{-7}$) might explain the higher frequency of severe eye complications in Caucasian cases, although this finding was not observed for other manifestations of the disease.

The implication of sex in the natural history of PXE has not been demonstrated to date. This has a specific impact on medical, esthetic, and social considerations because females had more severe skin lesions than males in our cohort. Conversely, males were at high risk for severe cardiac complications. This result might only reflect the well-known sex difference with respect to cardiovascular risk, which would be enhanced by the addition of PXE cardiac involvement.

Some guidelines for the management of patients with PXE have been proposed by the PXE international consortium (http://www.pxe.org) but have not been subjected to publication. Several therapeutic options have recently been raised,[32]

particularly for eye complications. In this context, our study might help clinicians to develop more personalized follow-up based on ethnic background, sex, and molecular status of their patients. Based on our results, we propose reinforcing cardiovascular follow-up in males and in cases with complete *ABCC6* haploinsufficiency, as well as ophthalmologic follow-up in Caucasians. We also propose that renal lithiases and strokes that have previously been shown with similar prevalence[33] be part of the complications to be sought in PXE clinical evaluation and included in a modified PS (**Supplementary Figure S1** online).

In conclusion, we herein present the largest series of PXE cases with complete clinical and molecular data screened in the Genetics Department of the Georges Pompidou European Hospital, France.

Our findings confirm that cardiac and gastric complications are rare; the main phenotype involves the skin and eyes, with a variable involvement of vascular manifestations. Renal and cerebral assessments of cases led us to confirm recurrent complications such as strokes and renal lithiases, which are not covered by the PS. We showed that sex, ethnic background, and number and type of detected variants influence the severity of the disease and/or the age at diagnosis. More importantly, we identified subgroups of patients with poorer functional prognoses based on their sex, molecular status, and ethnic background. Finally, we showed that the distribution of pathogenic variants along the ABCC6 protein is clearly biased toward the intracellular domains, with a particular enrichment in two specific subdomains.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/gim

ACKNOWLEDGMENTS

We thank all the PXE patients and their families for their kind collaboration. We acknowledge the contribution of all clinicians who gathered clinical data. We also thank the technicians of the genetics laboratory for their contribution to the *ABCC6* genetic analysis. This study was supported by Assistance Publique Hôpitaux de Paris, University Paris Descartes, and INSERM.

DISCLOSURE

The authors declare no conflict of interest.

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