MUTATION IN BRIEF

ABCC6 Mutations in Italian Families Affected by Pseudoxanthoma Elasticum (PXE)

Dealba Gheduzzi[1], Rita Guidetti[1], Claudia Anzivino[2], Patrizia Tarugi[1], Enza Di Leo[1], Daniela Quaglino[1], and Ivonne Pasquali Ronchetti[1]*

[1]Dept. Biomedical Sciences, University of Modena and Reggio Emilia, Modena, Italy; [2]Dept. Medicine and Medical Specialities, University of Modena and Reggio Emilia, Modena, Italy

*Correspondence to: Prof. I.Pasquali Ronchetti-Dept. Biomedical Sciences, University of Modena and Reggio Emilia-Via Campi 287, 41100 Modena, Italy; Tel.: 0039-059-2055418; Fax: 0039-059-2055426; E-mail: ronchetti.ivonne@unimore.it

Grant sponsor: PXE International; Grant sponsor: Fondazione Cassa di Risparmio di Modena; Grant sponsor: MIUR, Grant number: MM06178355; Grant sponsor: Telethon Foundation ONLUS, Grant number: E0696.

Communicated by Claude Ferec

Pseudoxanthoma elasticum (PXE) is a genetic disorder, characterized by cutaneous, ocular and cardiovascular clinical symptoms, caused by mutations in a gene (*ABCC6*) that encodes for MRP6 (Multidrug Resistance associated Protein 6), an ATP-binding cassette membrane transporter. The *ABCC6* gene was sequenced in 38 unrelated PXE Italian families. The mutation detection rate was 82.9%. Mutant alleles occurred in homozygous, compound heterozygous and heterozygous forms, however the great majority of patients were compound heterozygotes. Twenty-three different mutations were identified, among which 11 were new. Fourteen were missense (61%); five were nonsense (22%); two were frameshift (8.5%) and two were putative splice site mutations (8.5%). The great majority of mutations were located from exon 24 to 30, exon 24 being the most affected. Among the others, exons 9 and 12 were particularly involved. Almost all mutations were located in the intracellular site of MRP6. A positive correlation was observed between patient's age and severity of the disorder, especially for eye alterations. The relevant heterogeneity in clinical manifestations between patients with identical *ABCC6* mutations, even within the same family, seems to indicate that, apart from PXE causative mutations, other genes and/or metabolic pathways might influence the clinical expression of the disorder. © 2004 Wiley-Liss, Inc.

KEY WORDS: Pseudoxanthoma elasticum; genetic disorder; *ABCC6*; MRP6; elastic fibre

INTRODUCTION

Pseudoxanthoma elasticum (PXE, MIM# 177850; MIM# 264800) is an inherited disorder characterised by mineralization of elastic fibres and abnormalities of collagen and matrix constituents in the soft connective tissues of all organs examined so far (Gheduzzi et al., 2003). Clinical manifestations mainly consist of coalesced papules and laxity in the flexural areas of skin, retinal angioid streaks and recurrent hemorrhage and vessel alterations similar to those in atherosclerosis (Neldner and Struck, 2002).

The gene responsible for PXE (*ABCC6*; MIM# 603234) is on chromosome 16p13.1 (Le Saux et al., 1999). It

Received 18 May 2004; accepted revised manuscript 13 August 2004.

DOI: 10.1002/humu.9284

consists of 31 exons spanning approximately 73kb and encodes for a protein (MRP6, Multidrug Resistance associated Protein 6) belonging to the ATP-binding cassette sub-family C of membrane transporters (Bergen et al., 2000; Le Saux et al., 2000; Ringpfeil et al., 2000). The precise localization in human tissues and the physiological role of MRP6 are still unknown (Scheffer et al., 2002). Recent reports suggest its implication in the cell extrusion of glutathionated metabolites (Belinsky et al., 2002; Ilias et al., 2002).

To date, more than 60 causative mutations for PXE have been reported (Hu et al., 2003; Chassaing et al., 2004). Irrespective of the geographic origin of affected individuals, the great majority of mutations have been found between exons 24 and 30 of the *ABCC6* gene (Le Saux et al., 2001; Meloni et al., 2001; Pulkkinen et al., 2001). This would suggest that the C-terminal end of the protein is of paramount importance for its function. However, one cannot exclude the possibility that the high frequency of mutations at the C-terminal of *ABCC6*/MRP6 reflects a founder effect (Torrington and Viljoen, 1991; Le Saux et al., 2002).

This study has been designed to identify mutations in the Italian population, to compare data with those obtained in families from other European Countries, and to correlate mutations to the clinical phenotype of affected individuals.

MATERIAL AND METHODS

Patients and Sampling

The coding regions and the intron/exon boundaries of *ABCC6* gene have been analysed in 54 Italian PXE patients. Controls were 50 unrelated healthy subjects of Italian origin. All subjects gave their written informed consent and the study was approved by the ethical Committee of the Faculty of Medicine of the University of Modena and Reggio Emilia.

Clinical evaluation

The diagnosis of PXE was made on dermatological and/or ophtalmological evaluations, followed by histological confirmation of calcified elastic fibres positive to the von Kossa stain in skin biopsies. PXE clinical manifestations met the previously published consensus criteria (Christiano et al., 1992). The majority of patients came to our laboratory with recent haematological evaluation, fluorescein angiogram of the fundus oculi, Doppler ultrasonography of the peripheral arteries, as well as echocardiogram.

Molecular analysis

Genomic DNA was isolated from whole blood (QIAamp blood kit, Qiagen), according to a standard procedure.

Primers and polymerase chain reaction

Primers for PCR amplification of all 31 exons and intron-exon junctions of *ABCC6* were synthesised as previously reported (Wang et al., 2001). To distinguish between *ABCC6* and pseudogene sequences, specific primers for exons 1-9 were used (Pulkkinen et al., 2001). The recognition of known large deletions involving exon 15 and exons 23-29 of ABCC6 was performed by PCR amplification of the regions involved as previously described (Le Saux et al., 2001). PCR conditions were: 94°C for 4min followed by 30 cycles at 94°C for 30 s/48-62°C for 30 s/72°C for 30 s with a final elongation at 72°C for 5 min. PCR products were purified by using High Pure PCR Product Purification Kit (Roche Diagnostics).

DNA sequencing

The PCR products were directly sequenced using an Applied Biosystems 3100 DNA sequencer, with ABI PRISM BigDye terminator Cycle Sequencing Kit (Applied Biosystems). The criteria previously reported (Le Saux et al., 2001) were adopted to define the sequence variants as causative of PXE. A panel of 100 alleles was used as control to confirm new mutations as causative of PXE.

RT-PCR

Total RNA was extracted from dermal fibroblasts cultured in vitro, by using Rneasy MINIKIT (Qiagen). First strand cDNA was sinthesized by RT-PCR by SuperScript^{TM} II RT Kit (Invitrogen). To amplify the exon 17-exon

19 region we used the following primers: 5'-TGGACCCACCCTGGCTGGAG-3' (forward primer in exon 17) and 5'GGTAGGAACCCATCTCTGCGAT-3' (reverse primer in exon 19). The amplification conditions were those already described, with an annealing temperature of 63°C. PCR products were eluted from the gel and purified by using Jet Quick Gel Extraction Spin Kit (Genomed GmbH) and were directly sequenced.

Haplotype analysis

A limited haplotype analysis was performed for mutations identified in homozygous state in at least two families, using twelve intragenic single-nucleotide polymorphisms (c.1233T>C, c.1245G>A, c.1841T>C, c.1890C>G, c.1896C>A, c.2490T>C, c.3803G>A, c.1868-92delG, c.1868-90G>T, c.1868-57G>A, c.4404-76A>G, c.4404-31G>A).

Mutation Nomenclature

All mutations are described according to mutation nomenclature (den Dunnen and Antonorakis, 2000; den Dunnen and Paalman, 2003; and at http://www.hgvs.org/mutnomen/). Nucleotide numbers are derived from cDNA *ABCC6* sequences (GenBank accession no. NM_00171.2).

Statistical analysis

Typical PXE clinical manifestations were scored as follows:

a) Skin: 1 – yellow papules on flexural areas; 2 – plaques of coalesced papules; 3 – laxity and redundancy;
b) Eyes: 1 – peau d'orange pigmentation; 2 – angioid streaks; 3 – retinal haemorrhages; 4 – disciform scarring;
c) Vessels: 1 – weak or absent pulses; 2 – intermittent claudication; 3 – occlusion;
d) Heart: 1 – angina pectoris; 2 – mitral valve prolapse; 3 – myocardial infarction; 4 – symptoms severe enough to warrant by-pass or angioplasty;
e) Gastro-intestinal tract: 1 – bleeding; 2 – intestinal angina.

Data were expressed as mean values ± standard deviation and compared by non parametric statistical tests, i.e. Mann Whitney. Significance of data was taken at $p \leq 0.05$. A linear regression analysis was performed to correlate age and clinical score.

RESULTS

The *ABCC6* gene was sequenced in 54 PXE patients belonging to 41 families, among which 37 were unrelated families and 4, having a common ancestor in the eighteen century (table 1), were considered as one family. PXE families were from northern (57.9%), central (26.3%) and southern (15.8%) Italy. The different distribution reflects the easiness of sample collection and not the prevalence of PXE in different parts of the country. In almost all families, pedigree analysis suggested autosomal recessive inheritance.

Sequence variants found in *ABCC6* gene

Mutations were identified in 63 of the 76 alleles examined (mutation detection rate = 82.9%). Among the 13 unidentified alleles, 5 were the second mutation in 5 families (6 patients), and the remaining 8 belonged to 4 families (5 patients) in whom we were not able to identify any mutation in the *ABCC6* gene. Table 1 reports families and individuals in which PXE causative mutations have been identified on one or both *ABCC6* alleles. Mutant alleles occurred in all combinations including homozygous, compound heterozygous and heterozygous forms. The great majority of patients were compound heterozygotes.

Twenty-three different *ABCC6* mutations were identified (table 2), including 14 missense (61%), 5 nonsense (22%), 2 putative splice site mutations (8.5%), 2 frameshift mutations (8.5%). Nonsense mutations were found in 50% of the alleles either in homozygous or in heterozygous state.

Table 1. PXE-causative Mutations Recognized (on one and both alleles) in Italian Patients

Family/ Proband	Affected subjects	Age / gender	Clinical score	Tot	Mutations* Allele 1	Allele 2	Mutation type
I-3097	001	32 F	S2,E2	4	p.R518Q	p.T1130M	missense
	002	36 M	S3,E2,V2,C2	9	p.R518Q	p.T1130M	
I-3013	001	46 F	S1,E3	4	p.R1339C	None found	
I-3094	001	57 F	S2,E2	4	p.C440G	p.P1346S	
I-3103	001	57 M	E2	2	p.V810M	p.R1114C	
I-3076	001	57 F	S2,E4,V3	9	p.R1339C	p.R1339C	
I-3016	001	69 F	S3,E2,V2	7	p.N411K	p.R1138Q	
I-3082	001	23 M	S1,E2	3	p.R518Q	p.R1141X	missense + nonsense
I-3074	001	27 F	S2,E2	4	p.T364R	p.R518X	
I-3015	001	27 F	S2,E3	5	p.Q378X	p.R600G	
I-3062	001	45 M	S2,E4,V2	8	p.R1141X	p.E1400K	
I-3067	001	50 F	S1	1	p.R1275X	p.E1400K	
	002	60 F	S3,E3	6	p.R1275X	p.E1400K	
	003	66 F	S2,E2	4	p.R1275X	p.E1400K	
I-3027	001	61 F	S3,E2	4	p.R518Q	p.R1141X	
	002	63 F	S3,E4,V3	10	p.R518Q	p.R1141X	
I-3056**	001	23 F	S3,E2	5	p.R1141X	p.R1141X	nonsense
	002	32 M	S2,E2	4	p.R1141X	p.R1141X	
I-3057**	001	27 F	S1,E2	3	p.R1141X	p.R1141X	
	002	31 M	S3,E2	5	p.R1141X	p.R1141X	
I-3045	001	28 M	S1,E2	3	p.R1141X	None found	
	002	32 F	S3,E2,V1	6	p.R1141X	None found	
I-3107	001	29 M	S2,E1	3	p.R1030X	p.R1141X	
I-3073	001	31 F	S3,E2	5	p.R1141X	p.R1141X	
I-3111	001	32 F	S1,E2	3	p.R1141X	p.R1141X	
I-3090	001	34 F	S2,E1	3	p.R1141X	p.R1141X	
I-3001	001	37 F	S3,E2,V2	7	p.R1030X	None found	
I-3007**	001	40 F	S2,E2	4	p.R1141X	p.R1141X	
	002	48 F	S1,E2	3	p.R1141X	p.R1141X	
I-3114	001	40 M	S2,E2,V3,C1,G2	10	p.R518X	p.R518X	
I-3054	001	44 F	S2,E3	5	p.R518X	p.R518X	
I-3055**	001	47 F	S3,E4,C2,G1	10	p.R1141X	p.R1141X	
	002	50 F	S3,E3	6	p.R1141X	p.R1141X	
I-3017	001	50 F	S3,E4,V3,C2	12	p.R518X	p.R1030X	
	002	52 F	S3,E4,V3	10	p.R518X	p.R1030X	
	003	55 F	S3,E2	5	p.R518X	p.R1030X	
I-3100	001	52 M	S3,E3	6	p.Q378X	p.Q378X	
I-3051	001	53 F	S3,E4,V2	9	p.R1141X	p.R1141X	
I-3034	001	53 M	S3,E4,V3	10	p.R1141X	p.R1141X	
I-3093	001	57 F	S3,E3,V2,C3	11	p.R518X	None found	
I-3087	001	57 F	S3,E4,V2,C2	11	p.Q378X	p.Q378X	
I-3040	001	60 F	S3,E4,V2	9	p.R1141X	None found	
I-3033	001	62 F	S3,E4	7	p.R1141X	p.R1141X	
I-3026	001	36 F	S3,E2,G1	6	p.R518X	c.2248-2_2248-1del	others
I-3024	001	40 F	S1,E2,V3	6	p.R518X	p.L1182PfsX96	
I-3072	001	41 F	S2,E2,C2	6	p.M1127T	c.3736-1G>A	
I-3002	001	50 F	S3,E2	5	p.A820P	c.3736-1G>A	

Family/ Proband	Affected subjects	Age / gender	Clinical score	Tot	Mutations* Allele 1	Allele 2	Mutation type
	002	57 F	S2,E4	6	p.A820P	c.3736-1G>A	
I-3008	001	53 F	S2,E2,C1	5	p.M1440CfsX24	p.M1440CfsX24	

Patients are identified by an international code: I = Italian, 3001 = family number (European patients are numerated from 3000), 001 = subject number. Patients have been divided for type of mutation and, in each group, are listed by increasing age. Age, gender and clinical manifestations are reported. Progressive scores were given accordingly to the severity of clinical manifestations in each organ (see method section), where S is for skin; E for eye; V for vessel; C for heart; G for gastro-intestinal tract. The total score is also reported.

***** GenBank accession no. NM_001171.2. For cDNA numbering +1 corresponds to the A of the ATG translation initiation codon.

** Families with a common ancestor in the XVIIIth century were considered as one for mutation frequency analysis.

Table 2. *ABCC6*/MRP6 Mutations Found in Italian PXE Patients

Number of families	INTRON	EXON	cDNA*	PROTEIN*	References
1		9	c.1091C>G	p.T364R	Pulkkinen et al., 2001
3		9	c.1132C>T	p.Q378X	Pulkkinen et al., 2001; Cai et al., 2001
1		**10**	**c.1318T>G**	**p.C440G**	Present study
1		10	c.1233T>G	p.N411K	Le Saux et al., 2001
7		12	c.1552C>T	p.R518X	Meloni et al., 2001; Chassaing et al., 2004
3		12	c.1553G>A	p.R518Q	Le Saux et al., 2001; Chassaing et al., 2004
1		**14**	**c.1798C>T**	**p.R600G**	Present study
1	**17**		**c.2248-2_2248-1del**	**-**	Present study
1		**19**	**c.2428G>A**	**p.V810M**	Present study
1		**19**	**c.2458G>C**	**p.A820P**	Present study
3		23	c.3088C>T	p.R1030X	Le Saux et al., 2001
1		**24**	**c.3340C>T**	**p.R1114C**	Present study
1		**24**	**c.3380C>T**	**p.M1127T**	Present study
1		24	c.3389C>T	p.T1130M	Chassaing et al., 2004; Gotting et al., 2004
1		24	c.3413G>A	p.R1138Q	Le Saux et al., 2000; Ringpfeil et al., 2000; Le Saux et al., 2001
13		24	c.3421C>T	p.R1141X	Bergen et al., 2000; Germain et al., 2000; Ringpfeil et al., 2000; Le Saux et al., 2001; Pulkkinen et al., 2001; Uitto et al., 2001; Hu et al., 2003 ; Gotting et al., 2004
1		**25**	**c.3544_3544dupC**	p.L1182PfsX96	Present study
2	26		c.3736-1G>A	-	Ringpfeil et al., 2000; Le Saux et al., 2001
1		**27**	**c.3823C>T**	**p.R1275X**	Present study
2		28	c.4015C>T	p.R1339C	Le Saux et al., 2001
1		**28**	**c.4036C>T**	**p.P1346S**	Present study
2		29	c.4198G>A	p.E1400K	Chassaing et al., 2004
1		**30**	**c.4318_4318delA**	**p.M1440CfsX24**	Present study

The number of families in which a specific mutation was found (in heterozygous and in homozygous state) is reported. New mutations are in bold character.

***** GenBank accession no. NM_001171.2. For cDNA numbering +1 corresponds to the A of the ATG translation initiation codon.

In the PXE patients examined, exon 24 of the *ABCC6* gene was the most affected one, and c.3421C>T (p.R1141X) was the most frequent mutation, being homozygous in 7 of 38 families (18.4%), and heterozygous in another 6 unrelated families (15.8%), including the 2 families (3 patients) in whom it was the only detected mutation. Therefore, the p.R1141X mutation was present in 26.3% of all the alleles examined (20/76). Exon 24 was affected by other types of mutations [c.3340C>T (p.R1114C), c.3380C>T (p.M1127T), c.3389C>T (p.T1130M), c.3413G>A (R1138Q)] in four families, for a total of 17 out of 38 families (44.7%) and for a total of 24 alleles over the 76 examined (31.6%). Exons 9 and 12 were rather frequently affected (table 2), mutations being found in 7.9% and 15.8% of the alleles, respectively. In exon 9, c.1132C>T (p.Q378X) mutation was found in homozygosity in two families and in compound heterozygous in one family. In exon 12, mutation c.1552C>T (p.R518X) was homozygous in two families and heterozygous in other 5 families, and mutation c.1553G>A (p.R518Q) was heterozygous in 3 unrelated families. In order to identify a possible founder origin of recurrent disease-associated alleles, a limited haplotype analysis was performed in 11 unrelated families carrying the same mutation (p.Q378X, p.R518X, p.R1141X) in homozygous state (data not shown). The analysis of the two families with the p.Q378X mutation revealed different alleles, identical-by-state. The analysis of the two families with the p.R518X mutation revealed common haplotypes, suggesting a possible consanguinity. The analysis of the seven families with the p.R1141X mutated allele showed common haplotypes, suggesting the presence of a common ancestor. Comparison of the analysis of 9 intragenic single-nucleotide polymorphisms, evaluated in Italian as well as in French patients (Chassaing et al., 2004), showed identical alleles, suggestive for a founder origin of p.R1141X mutation in European PXE patients. The same comparison, in the case of p.R518X mutation, revealed different alleles, between Italian and French patients, identical-by state.

Figure 1 shows the location, on the MRP6 protein, of the PXE-causative mutations found in Italian families. Almost all mutations involved amino acid residues located in the intracellular site of the protein; moreover, 52% of mutations were located in the C-terminal of the MRP6 protein. Among missense mutations, two were located in the first part of the protein, whereas the remaining 6 involved amino acid changes in the second half of MRP6.

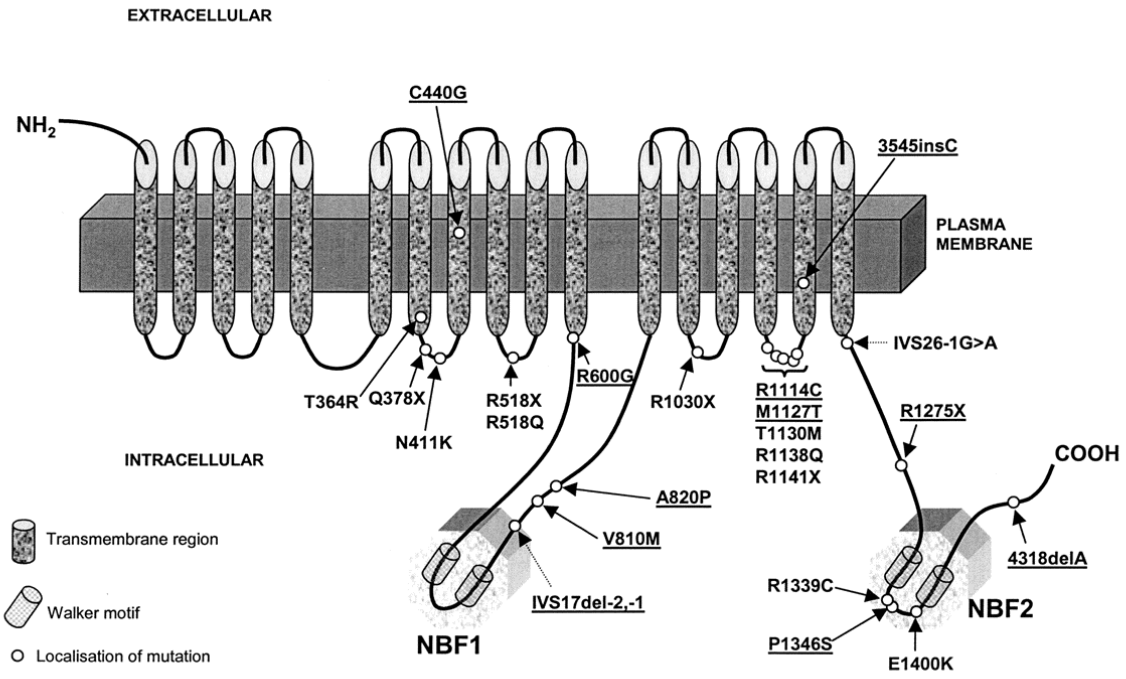

Figure 1. Schematic representation of the MRP6 protein and of the spectrum of mutations associated with PXE in the Italian families examined in the present study. New mutations are underlined. Mutations already described in the literature and not found in this study have not been reported.

Novel mutations

In the present study, 11 novel PXE-causing mutations were identified (table 2): 7 missense, 1 nonsense, 2 frameshift and 1 splice-site. The nonsense and the frameshift mutations are predicted to result in a premature termination of MRP6 and may be undoubtely considered causative of PXE. The 7 missense mutations can be regarded as disease-associated alleles since they were not found in our control panel of 100 alleles.

The majority of missense mutations would probably result in alterations of the MRP6 conformation [c.1318T>G (p.C440G), c.1798C>T (p.R600G), c.2428G>A (p.V810M), c.2458G>C (p.A820P), c.3340C>T (p.R1114C), c.3380C>T (p.M1127T)], whereas c.4036C>T (p.P1346S) mutation would probably cause a loss of function of MRP6, since previous studies have shown that missense mutations located in the second NBF (Nucleotyde Binding Fold) of MRP6 are able to completely abolish the transport activity of the protein (Ilias et al., 2002). As far as the amino acid changes, 6 missense mutations cause amino acid substitutions that lead to the introduction of a residue with physico/chemical properties different from the replaced amino acid. All but one mutation (p.M1127T) involve residues highly conserved in the MRP6 protein among different species (Mus musculus; Rattus norvegicus) and all but two mutations (p.C440G; p.M1127T) involve residues rather conserved in the C subfamily of ABC membrane transporters.

The nonsense mutation [c.3823C>T (p.R1275X)] and the two frameshift mutations [c.3544_3544dupC (p.L1182PfsX96), c.4318_4318delA (p.M1440CfsX24)] are predicted to result in the production of a truncated protein.

Finally, a new mutation affecting a consensus splice-site was found (c.2248-2_2248-1del).

Study of the effect of c.2248-2_2248-1del mutation

In order to evaluate the effect of c.2248-2_2248-1del mutation, found in a patient in compound heterozygous state, we performed a RT-PCR reaction of the region involved. The amplification of control cDNA resulted in a PCR product of 363bp, whose sequence corresponded to the expected *ABCC6* region (exon 17-19). The amplification of the same region of patient's cDNA gave rise to the production of two distinct PCR products, of 363 and 195bp. The direct sequencing of the 363bp fragment resulted in a normal *ABCC6* sequence, whereas the analysis of the 195bp PCR product revealed a sequence containing exons 17 and 19, but totally lacking of exon 18. This finding show that the deletion of the di-nucleotide AG, belonging to the 3' end of intron 17, and constituting the acceptor splicing site, results in skipping of exon 18 and in the in-frame junction of exon 17 and 19. The consequence, at protein level, is the absence of 56 amino acids from position 750 to 805, corresponding to the cytoplasmic region just after the NBF1 of MRP6.

Genotype-phenotype correlation

Table 1 illustrates the relationships between mutations and clinical phenotypes of unrelated patients and of patients within the same family. Eyes and skin were the organs more severely involved, independently of the type of mutation. Nonsense mutations seemed to be more frequently associated with a generalised involvement. A significant positive correlation ($p<0.02$) between patients' age and severity of clinical manifestations was observed in all PXE patients. In particular, by considering patients homozygous for the most recurrent p.R1141X mutation, that is associated with the almost complete absence of MRP6 (Le Saux et al., 2001; Hu et al., 2003), the correlation between total clinical score and age was highly consistent ($p<0.01$). However, skin alterations, by themselves, did not appear to increase significantly with age ($p=0.25$), whereas a significant age-dependent increase of eye manifestations was observed ($p<0.001$). In the older patients cardiovascular symptoms were also noted.

In families in which more than one member is affected by PXE, a high clinical heterogeneity was evident among close relatives, independent of the type of mutation.

DISCUSSION

***ABCC6* mutations**

The direct sequencing of the *ABCC6* gene in 38 Italian families with one or more members affected by PXE, pointed out 23 different *ABCC6* mutations, 11 of which were new variants, not previously described, with a mutation detection rate of 82.9%. Combining our data with those present in the literature to date, the total number

of PXE-causative mutations is 79. Most individuals were compound heterozygotes. Some were recurrent mutations (p.Q378X, p.R518X, p.R518Q, p.R1141X), however the majority of mutations were sporadic variants. The most frequent p.R1141X PXE-related allele shared a common haplotype identical-by-descent in seven Italian families homozygous for this mutation. Moreover, comparison of haplotype analyses in Italian and in French PXE families (Chassaing et al., 2004) may suggest the presence of a common ancestor. Our results revealed a distribution of mutations along the *ABCC6* gene similar to those published for other European Countries (Le Saux et al., 2001; Hu et al., 2003; Chassaing et al., 2004). The majority of mutations are located in the C-terminal domains of MRP6 and reside in cytoplasmatic domains of the protein. Moreover, 50% of the missense mutations occurred within two domains particularly affected by PXE-causing mutations: the large intracellular loop encoded by exon 24, and the second nucleotide binding fold (NBF2) of MRP6.

Genotype-phenotype correlation

The present data clearly indicates that there is an age-dependent progression in the number of organs involved and the severity of clinical manifestations. These data agree with recent findings that all organs in PXE patients exhibit extracellular matrix alterations (Gheduzzi et al., 2003) that become clinically more relevant with time.

The group of patients analysed in the present study is rather small to delineate a precise genotype-phenotype correlation, however interesting data emerged from the analysis of 10 families in which two or three siblings, of comparable age and life-style, were affected by the disorder. The evaluation of the clinical involvement in each organ revealed that, irrespective of the type of mutation, there was a very high heterogeneity within the same family. In particular, three sisters, with nonsense mutations on both alleles, manifested severe involvement of skin and eyes, whereas the cardiovascular system was affected in only two of them. As a further example, patient I-3055-001, who was also a strong smoker, was the only PXE patient homozygous for the p.R1141X mutation with heart and gastro-intestinal alterations, whereas her sister had only eye and skin lesions, underlining the importance of exogenous factors that may interfere with elastin deposition and degradation. For instance, it is known that the cigarette smoke is deleterious for mesenchymal cells (Ishii et al., 2001; Santos et al., 2002) and for the elastic system in general, as it affects lysyl oxidase activity (Laurent et al., 1983). However, little is known about the mineralization process and in particular what may induce elastic fibres to mineralise in PXE. It can be hypothesize that *ABCC6*/MRP6 deficiency would induce retention of cellular products which affect fibroblast metabolism (Boraldi et al., 2003) causing, as final consequence, extracellular matrix alterations (Neldner and Struk, 2002; Gheduzzi et al., 2003). Therefore, the relevant heterogeneity in clinical manifestations between relatives may suggest that, apart from PXE causative mutations, other genes and/or metabolic pathways that may have overall influence on the clinical expression of the disorder, as also suggested in a 20% of beta-thalassemia patients with PXE-like alterations (Baccarani-Contri et al., 2001). Furthermore, a large number of polymorphisms have been found in the *ABCC6* gene that are not disease associated, but which could have a functional effect, similar to what observed for other ABC transporters (Ito el al., 2001; Rowntree and Harris, 2003). Interestingly, it has been observed that a particular *ABCC6*/MRP6 polymorphism, called c.3803G>A (p.R1268Q), is associated with reduced amount of plasma triglycerides and higher plasma HDL cholesterol (Wang et al., 2001). Although *ABCC6*/MRP6 does not seem to be involved in lipid metabolism, this finding could be relevant for the expression of clinical manifestations in subjects bearing a PXE causative mutation, at least for the alterations affecting the cardiovascular system. Another possible explanation is that the *ABCC6* gene might not be the only participant in the PXE pathogenesis, and that other genetic factors, such as alternative genomic loci containing modifier genes, as observed in cystic fibrosis (Rowntree and Harris, 2003), could be also involved.

ACKNOWLEDGMENTS

Authors are grateful to the PXE-Italia Association, to PXE patients and families for their helpful collaboration, to Mrs. Sharon Terry and to Dr. Lionel Bercovitch for reading the manuscript.

REFERENCES

Baccarani-Contri M, Bacchelli B, Boraldi F, Quaglino D, Taparelli F, Carnevali E, Francomano MA, Seidenari S, Bettoli V, De Sanctis V, Pasquali-Ronchetti I. 2001. Characterization of pseudoxanthoma elasticum-like lesions in the skin of patients with beta-thalassemia. J Am Acad Dermatol 44: 33-39.

Belinsky MG, Chen ZS, Shchaveleva I, Zeng H, Kruh GD. 2002. Characterization of the drug resistance and transport properties of multidrug resistance protein 6 (MRP6, *ABCC6*). Cancer Res 162: 6172-6177.

Bergen AA, Plomp AS, Shuurman EJ, Terry S, Breuning M, Dauwerse H, Swart J, Kool M, van Soest S, Baas F, ten Brink JB, de Jong PT. 2000. Mutations in *ABCC6* cause pseudoxanthoma elasticum. Nat Genet 25: 228-231.

Boraldi F, Quaglino D, Croce MA, Garcia-Fernandez MI, Tiozzo R, Gheduzzi D, Bacchelli B, Pasquali-Ronchetti I. 2003. Multidrug resistance protein-6 (MRP6) in human dermal fibroblasts. Comparison between cells from normal subjects and from Pseudoxanthoma elasticum patients. Matrix Biol 22: 491-500.

Cai L, Lumsden A, Guenther UP, Neldner SA, Zach S, Knoblauch H, Ramesar R, Hohl D, Callen DF, Neldner KH, Lindpaintner K, Richards RI, Struk B. 2001. A novel Q378X mutation exists in the transmembrane transporter protein *ABCC6* and its pseudogene: implications for mutation analysis in pseudoxanthoma elasticum. J Mol Med 79: 536-546.

Chassaing N, Martin L, Mazereeuw J, Barrie L, Nizard S, Bonafe JL, Calvas P, Hovnanian A. 2004. Novel *ABCC6* mutations in pseudoxanthoma elasticum. J Invest Dermatol 122: 608-613.

Christiano AM, Lebwohl MG, Boyd CD, Uitto J. 1992. Workshop on pseudoxanthoma elasticum: molecular biology and pathology of the elastic fibers. J Invest Dermatol 99: 660-663.

den Dunnen JT, Antonarakis SE. 2000. Mutation nomenclature extensions and suggestions to describe complex mutations: a discussion. Hum Mut 15:7-12.

den Dunnen JT, Paalman MH. 2003. Standardizing mutation nomenclature: Why bother? Hum Mut 22:181-182.

Germain DP, Remones V, Perdu J, Jeunemaitre X. 2000. Identification of two polymorphisms (c189G>C; c190T>C) in exon 2 of the human MRP6 gene (*ABCC6*) by screening of Pseudoxanthoma elasticum patients: possible sequence correction? Hum Mutat 16: 449.

Gheduzzi D, Sammarco R, Quaglino D, Bercovitch L, Terry S, Taylor W, Pasquali-Ronchetti I. 2003. Extracutaneous ultrastructural alterations in pseudoxanthoma elasticum. Ultrastruct Pathol 27: 375-384.

Gotting C, Schulz V, Hendig D, Grundt A, Dreier J, Szliska C, Brinkmann T, Kleesiek K. 2004. Assessment of a rapid-cycle PCR assay for the identification of the recurrent c.3421C>T mutation in the *ABCC6* gene in pseudoxanthoma elasticum patients. Lab Invest 84: 122-130.

Hu X, Plomb A, Wijnholds J, ten Brink J, Van Soest S, Van Den Born LI, Leys A, Peek R, De Jong PTVM, Bergen AAB. 2003. *ABCC6*/MRP6 mutations: further insight into the molecular pathology of pseudoxanthoma elasticum. Eur J Hum Gen 11: 215-224.

Ilias A, Urban Z, Seidl TL, Le Saux O, Sinko E, Boyd CD, Sarkadi B, Varadi A. 2002. Loss of ATP-dependent transport activity in pseudoxanthoma elasticum-associated mutants of *ABCC6* (MRP6). J Biol Chem 277: 16860-16867.

Ishii T, Matsuse T, Igarashi H, Masuda M, Teramoto S, Ouchi Y. 2001. Tobacco smoke reduces viability in human lung fibroblasts: protective effect of glutathione S-transferase P1. Am J Physiol Lung Cell Mol Physiol 280: L1189-1195.

Ito K, Oleschuk CJ, Westlake C, Vasa MZ, Deeley RG, Cole SP. 2001. Mutation of Trp1254 in the multispecific organic anion transporter, multidrug resistance protein 2 (MRP2) (ABCC2), alters substrate specificity and results in loss of methotrexate transport activity. J Biol Chem 276: 38108-38114.

Laurent P, Janoff A, Kagan HM. Cigarette smoke blocks cross-linking of elastin in vitro. 1983. Am Rev Respir Dis 127: 189-192.

Le Saux O, Urban Z, Goring HH, Csiszar K, Pope FM, Richards A, Pasquali-Ronchetti I, Terry S, Bercovitch L, Lebwohl MG, Breuning M, van den Berg P, Kornet L, Doggett N, Ott J, de Jong PT, Bergen AA, Boyd CD. 1999. Pseudoxanthoma elasticum maps to an 820-kb region of the p13.1 region of chromosome 16. Genomics 62: 1-10.

Le Saux O, Urban Z, Tschuch C, Csiszar K, Bacchelli B, Quaglino D, Pasquali-Ronchetti I, Pope FM, Richards A, Terry S, Bercovitch L, de Paepe A, Boyd CD. 2000. Mutations in a gene encoding an ABC transporter cause pseudoxanthoma elasticum. Nat Genet 25: 223-228.

Le Saux O, Beck K, Sachsinger C, Silvestri C, Treiber C, Goring HH, Johnson EW, De Paepe A, Pope FM, Pasquali-Ronchetti I, Bercovitch L, Marais AS, Viljoen DL, Terry SF, Boyd CD. 2001. A spectrum of *ABCC6* mutations is responsible for pseudoxanthoma elasticum. Am J Hum Genet 69: 749-764.

Le Saux O, Beck K, Sachsinger C, Treiber C, Goring HH, Curry K, Johnson EW, Bercovitch L, Marais AS, Terry SF, Viljoen DL, Boyd CD. 2002. Evidence for a founder effect for pseudoxanthoma elasticum in the Afrikaner population of South Africa. Hum Genet 111: 331-338.

Meloni I., Rubegni P., De Aloe G., Bruttini M., Pianigiani E., Cusano R., Seri M., Mondillo S., Federico A., Bardelli A.M., Andreassi L., Fimiani M., Renieri A. Pseudoxanthoma elasticum: Point mutations in the *ABCC6* gene and a large deletion including also ABCC1 and MYH11. Hum.Mutat. 18(1): 85, 2001.

Neldner KH, Struk B. 2002. Pseudoxanthoma elasticum. In: Royce PM, Steinmann B, editors. Connective tissue and Its Heritable Disorders. Molecular, genetic and medical aspects. New York: Wiley Liss. p 561-583.

Pulkkinen L, Nakano A, Ringpfeil F, Uitto J. 2001. Identification of *ABCC6* pseudogenes on human chromosome 16p: implications for mutation detection in pseudoxanthoma elasticum. Hum Genet 109: 356-365.

Ringpfeil F, Lebwohl MG, Christiano AM, Uitto J. 2000. Pseudoxanthoma elasticum: mutations in the MRP6 gene encoding a transmembrane ATP-binding cassette (ABC) transporter. Proc Natl Acad Sci 97: 6001-6006.

Rowntree RK, Harris A. 2003. The phenotypic consequences of CFTR mutations. Ann Hum Genet 67: 471-485.

Santos S, Peinado VI, Ramirez J, Melgosa T, Roca J, Rodriguez-Roisin R, Barbera JA. 2002. Characterization of pulmonary vascular remodelling in smokers and patients with mild COPD. Eur Respir J 19: 632-638.

Scheffer GL, Hu X, Pijnenborg AC, Wijnholds J, Bergen AA, Scheper RJ. 2002. MRP6 (*ABCC6*/MRP6) detection in normal human tissues and tumors. Lab Invest 82: 515-518.

Torrington M, Viljoen DL. 1991. Founder effect in 20 Afrikaner kindreds with pseudoxanthoma elasticum. S Afr Med J 79: 7-11.

Uitto J, Pulkkinen L, Ringpfeil F. 2001. Molecular genetics of pseudoxanthoma elasticum: a metabolic disorder at the environment-genome interface? Trends Mol Med **7**: 13-17.

Wang J, Near S, Young K, Connelly PW, Hegele RA. 2001. *ABCC6* gene polymorphism associated with variation in plasma lipoproteins. J Hum Genet 46: 699-705.