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ORIGINAL INVESTIGATION

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Identification of ABCC6 pseudogenes on human chromosome 16p: implications for mutation detection in pseudoxanthoma elasticum

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Abstract Pseudoxanthoma elasticum (PXE), a heritable disorder affecting the skin, eyes, and the cardiovascular system, has recently been linked to mutations in the ABCC6 gene on chromosome 16p13.1. The original mutation detection strategy employed by us consisted of the amplification of each exon of the ABCC6 gene with primer pairs placed on the flanking introns, followed by heteroduplex scanning and direct nucleotide sequencing. However, this approach suggested the presence of multiple copies of the 5'-region of the gene when total genomic DNA was used as a template. In this study, we have identified two pseudogenes containing sequences highly homologous to the 5'-end of ABCC6. First, by the use of allele-specific polymerase chain reaction (PCR), two bacterial artificial chromosome (BAC) clones containing a putative pseudogene of ABCC6, designated as ABCC6-ψ1, were isolated from the human BAC library. Sequence analysis of ABCC6-ψ1 revealed it to be a truncated copy of ABCC6, which contains the upstream region and exon 1 through intron 9 of the gene. Secondly, a homology search of a high-throughput sequence database revealed the presence of another truncated copy of ABCC6, which was designated as ABCC6-ψ2, and which was shown to harbor upstream sequences and a segment spanning exon 1 through intron 4 of ABCC6. In addition to several nucleotide differences in the flanking introns and the upstream region, both pseudogenes contain several nucleotide changes in the exonic sequences, including stop codon mutations, which complicate mutation analysis in patients with PXE. Nucleotide differences in flanking introns between these two pseudogenes and ABCC6 allowed us to design allele-specific primers that eliminated the amplification of both pseudogene sequences by PCR and provided reliable amplification of ABCC6-specific sequences only. The use of allele-specific PCR has revealed, thus far, two novel 5'-end PXE mutations, 179del9 and T364R in exons 2 and 9, respectively, and several polymorphisms within the upstream region and exons 1–9 of ABCC6. These strategies facilitate comprehensive analysis of ABCC6 for mutations in PXE.

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Introduction

The ABCC6 gene encodes a multidrug resistance associated protein, MRP6, a member of the ATP-binding cassette (ABC) superfamily of membrane transporter proteins (Belinsky and Kruh 1999; Borst et al. 1999). This gene/protein system has recently been shown to be associated with the pathogenesis of pseudoxanthoma elasticum (PXE), a rare heritable disorder affecting the skin, the eyes, and the cardiovascular system (Neldner 1988; Ringpfeil et al. 2001a; Uitto et al. 2001). A spectrum of mutations, including missense and nonsense mutations, small insertions or deletions, and a recurrent 16.5-kb genomic deletion spanning exons 23–29, has been characterized in the ABCC6 gene in patients with PXE (Ringpfeil et al. 2000, 2001b; Struk et al. 2000; Bergen et al. 2000; Le Saux et al. 2000).

The ABCC6 gene has been localized to the short arm of chromosome 16, to a region containing the ABCC1 gene, which encodes the prototypic member of membrane transporter proteins, MRP1 (Le Saux et al. 1999; Cai et al. 2000). In addition to ABCC1, the region surrounding the ABCC6 locus at 16p13.1 contains the PM5 gene encoding a protein with unknown function, two identical copies of the NPIP gene encoding a nuclear pore interacting protein, and two polycystic kidney disease (PKD) pseudogenes (Le Saux et al. 1999; Cai et al. 2000). It has been reported that chromosome 16p contains regions of several duplicons of 20–45 kb in size (Loftus et al. 1999). These duplicons have been shown to include several copies of known genes, including PKD-like genes, a translation initiation factor gene (eIF3-p110), and a number of putative genes of unknown function.

The strategies employed by us for mutation detection in PXE have been based on the amplification of each exon of ABCC6 by the use of primer pairs placed on the flanking introns, followed by heteroduplex scanning by conformation sensitive gel electrophoresis (CSGE), and automated nucleotide sequencing (Ringpfeil et al. 2000). Surprisingly, the initial mutation screening of the 5'-end of the gene revealed frequent nucleotide changes, including stop-codon-causing mutations in exons 2 and 9; these mutations were present in the heterozygous state in every individual tested, including apparently healthy controls with no family history of PXE. We concluded, therefore, that more than one template was amplified from the region spanning exons 1–9 of ABCC6. In this work, we have characterized two ABCC6 pseudogenes (ABCC6-ψ1 and ABCC6-ψ2) on chromosome 16p and have utilized this information to improve the mutation detection in PXE by the design of allele-specific primers that eliminate the amplification of both pseudogene sequences. Employing this strategy, we have identified two novel pathogenetic mutations in ABCC6 in patients with PXE, and several ABCC6-specific polymorphisms in the 5'-end of the gene.

Materials and methods

Mutation detection

DNA from PXE patients, their family members, and normal healthy controls was isolated from peripheral blood by standard methods (Sambrook et al. 1989). Mutation detection consisted of the amplification of each exon of ABCC6 with primers placed on the flanking intronic sequences. The polymerase chain reaction (PCR) products were then analyzed on 1.5%–2% agarose gels and subjected to CSGE as described previously (Ganguly et al. 1993; Körkkö et al. 1998). The PCR products forming heteroduplex bands were sequenced directly by the ABI 377 nucleotide sequencing system. The following primers were originally used for amplification of exons 2 and 9: E2-F, 5'-GATCCAAAAAGTTGCCTGGC-3'; E2-R, 5'-AAGACTTCACCAGGTTCCAG-3'; E9-F, 5'-AGGCACCTCCTCTCACCAGC-3'; E9-R, 5'-TCCTGGCTGGGAAGACCTGC-3'. The PCR consisted of 1×PCR buffer, 1×Q-buffer, and 1.25 U *Taq* polymerase (Qiagen), 200 μM nucleotide mix, 15 pmol each primer, and 100 ng genomic DNA, in a 50-μl total reaction volume. The amplification conditions were 95°C for 3 min, followed by 38 cycles of 95°C for 45 s, 60°C for 45 s, and 72°C for 45 s, and one cycle of 72°C for 10 min.

Verification of mutations and polymorphisms

Restriction enzyme digestions were used for the verification of mutations in PXE families and for the demonstration of their absence in more than 100 normal healthy controls. Specifically, *Nci*I restriction enzyme was used for the detection of mutation 179del9, *Bst*NI for the detection of mutation G1354R, and *Bsi*YI for mutation R1141X. For the detection of the mutation T364R, the following mismatch primer was used together with E9-R primer (see below): 5'-GATGTTCCTCTCAGCCTGCCTGCCAA-3', in which an A at position 1988 was substituted by a C (underlined), resulting in a restriction enzyme site for *Sty*I in the mutant allele.

Reverse transcription/PCR procedure

Total RNA was extracted from cultured cells by the use of the Trizol reagent method, and first-strand cDNA synthesis was performed by the use of MMLV-reverse transcriptase according to the manufacturer's recommendations (Gibco-BRL). The following primers for PCR were used: cE1-F, 5'-ACCCACGACGACAGAAGGCGCCGA-3'; cE1.1-F, 5'-ATGGCCGCGCCTGCTG-AGCCCTGC-3'; cE4-R, 5'-GCAAGACAAAGCAGAGAAGC-3'; cE7-R, 5'-TCTC-CCA-AGCGACCAGAGG-3'; cE8-R, 5'-CTCGGTCTCTGGAGCCTT-CATGCC-3'; cE9-R, 5'-ATGGCCGACCGCAACCTCATCTGC-3'.

Characterization of bacterial artificial chromosome clones

The following pseudogene-specific reverse primer placed on exon 9, together with the primer E9-F (see above), was used for screening of the human bacterial artificial chromosome (BAC) library (Incyte Genomics): PS-R, 5'-CCGACCGCAGCCTCATCTA-3' (the pseudogene-specific nucleotides are underlined). DNA from the BAC clones was extracted with the Plasmid Maxi Kit, as recommended by the manufacturer (Qiagen), and BAC DNA (1–1.5 μg) was used for each sequencing reaction on the ABI 377 automated sequence analyzer; 20 ng BAC DNA was also used as a template for mapping the following markers by PCR amplification: D16S2813, D16S2745, D16S2594E, D16S2816, D16S2847, D16S2859, D16S737, D16S410, D16S501, D16S287, D16S499, D16S2887, D16S2799, D16S2572E, D16S3041, D16S403, D16S764, D16S3036, D16S79, D16S3127, D16S3017, D16S3103. The primer sequences were as reported previously (Cao et al. 1999; Cai et al. 2000; Genome Database, http://www.gdb.org).

Database search

Sequence homology search was performed by BLAST analysis of the high-throughput genomic sequence (htgs) database through National Center for Biotechnology Information (Altschul et al. 1997; www.ncbi.nlm.gov/blast)

Clinical material

Three affected individuals in Family 1 presented with a classical PXE phenotype, including characteristic skin lesions with increased skin folding and "peau de orange". They also had moderate to severe loss of visual acuity. They are the product of a consanguineous marriage (see pedigree below). The patient in Family 2 is a sporadic case without family history and with classical clinical features of PXE, established by skin biopsy.

Results

Presence of multiple copies of ABCC6 in the human genome

Evidence for the presence of multiple copies of ABCC6 sequences, i.e., putative pseudogenes, was originally noted by CSGE, which demonstrated multiple heteroduplex bands in all DNA samples from 18 unrelated PXE patients and from six healthy controls when PCR products spanning exons 1–9 were examined (see Fig. 1). The sequence analysis of these PCR products revealed the presence of two heterozygous nucleotide changes in exon 2, including 191G→A, designated as R64Q, and 196insT, which causes a frameshift and results in premature termination codon within exon 3 (Fig. 1A). Furthermore, sequence analyses revealed three nucleotide variants in exon 9; these were consistently found in a heterozygous state in the DNA of PXE patients and in normal unrelated controls. One of the variants, 1132C→T, resulted in a STOP codon at amino

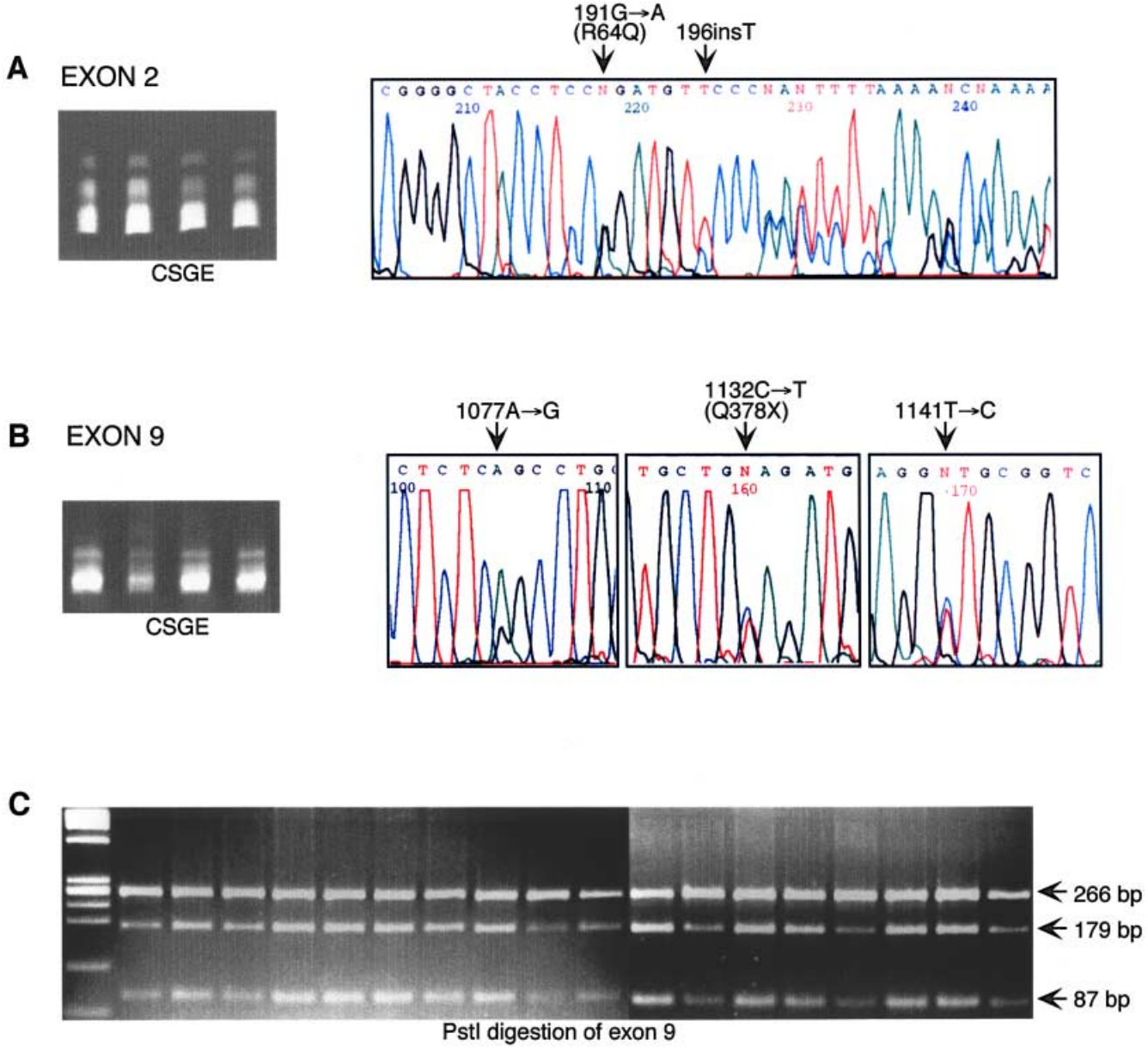

Fig. 1A–C Evidence for multiple copies of ABCC6 sequences. Amplification of exons 2 and 9 by PCR resulted in products that revealed multiple bands in all individuals by using heteroduplex analysis by CSGE (see **A, B**, *left*). Sequence analysis of the PCR products corresponding to exon 2 revealed two nucleotide changes, a G→A transition at position 191, which results in substitution of arginine by glutamine (R64Q), and an insertion of T at position 196, which results in a stop codon within exon 3 (**A**, *right*). Sequence analysis of the PCR products spanning exon 9 revealed three nucleotide changes, among which 1132C→T results in the substitution of a glutamine residue by a termination codon (Q378X). The two other changes, A→G at nucleotide position 1077 and T→C at nucleotide position 1141, are silent (**B**, *right*). The presence of Q378X in exon 9 was verified in 18 PXE patients (**C**) and in normal controls (not shown) by *Pst*I restriction enzyme digestion, which resulted in an undigested fragment of 266 bp in all samples studied, thereby reflecting the presence of the allele containing the Q378X mutation. The 179-bp and 87-bp bands correspond to the normal allele

acid position 378 (a mutation designated as Q378X; Fig. 1B). This mutation abolished a *Pst*I restriction enzyme site, which was used for the verification of this nucleotide change both in PXE patients and in normal controls (Fig. 1C). The two other nucleotide changes found in all DNA samples of exon 9, 1077A→G and 1141T→C, are silent (Fig. 1B). Based on these observations, we concluded that these and other nucleotide variations in the coding region spanning exons 1–9 (listed in Table 1) are not specific for ABCC6 but may be located in other copies of the gene.

Characterization of the ABCC6 pseudogenes

For the isolation of putative pseudogenes containing ABCC6 sequences, a primer pair selectively amplifying the pseudo-

Table 1 Nucleotide variations between ABCC6 and its two pseudogenes (*5'-UTR* 5' untranslated region)

Nucleotide position	Location	ABCC6	ABCC6-ψ1	ABCC6-ψ2
1–346	5'-UTR	a	g	g
1–339	5'-UTR	c	t	t
1–303	5'-UTR	a	a	c
1–116	5'-UTR	c	t	c
37–193	Intron 1	a	a	t
37–117	Intron 1	a	g	a
37–111	Intron 1	t	c	c
37–65	Intron 1	t	c	c
37–64	Intron 1	g	g	t
191	Exon 2	CGG (Arg)	CGG (Arg)	CAG (Gln)
196insT	Exon 2	–	+ (stop in exon 3)	–
219+38	Intron 2	t	g	g
219+40	Intron 2	c	g	g
230–286	Intron 2	a	a	g
230–95	Intron 2	t	c	t
230–63	Intron 2	c	c	t
230–60	Intron 2	c	c	t
232[a]	Exon 3	GCC (Ala)	GCC (Ala)	ACC (Thr)
373	Exon 4	GAG (Glu)	AAG (Lys)	GAG (Glu)
473	Exon 4	GCG (Ala)	GCG (Ala)	GTG (Val)
474+43	Intron 4	c	t	c
474+187	Intron 4	t	t	g
474+194	Intron 4	a	a	c
474+215	Intron 4	g	g	c
474+240	Intron 4	a	t	a
475–22	Intron 4	t	c	–
601–94	Intron 5	c	t	–
601–89	Intron 5	a	c	–
793[a]	Exon 7	AGG (Arg)	GGG (Gly)	–
794+36	Intron 7	a	c	–
794+78	Intron 7	a	c	–
794+95	Intron 7	t	c	–
794+154	Intron 7	t	c	–
794+181	Intron 7	a	g	–
794+182	Intron 7	g	c	–
794+198	Intron 7	g	a	–
795–51	Intron 7	c	t	–
795–117	Intron 7	c	t	–
855	Exon 8	ACC (Thr)	ACT (Thr)	–
999–269	Intron 8	c	g	–
1077	Exon 9	TCA (Ser)	TCG (Ser)	–
1132	Exon 9	CAG (Gln)	TAG (STOP)	–
1141	Exon 9	TTG (Leu)	CTG (Leu)	–
1176+59	Intron 9	g	a	–
1176+151	Intron 9	g	t	–
1176+178	Intron 9	t	c	–

[a]These positions are also polymorphisms in ABCC6 (see Table 3)

gene was designed based on the sequence differences within exon 9 (see Table 1). This primer pair, E9–F/PS–R, was used to amplify human genomic DNA, and sequencing of the PCR product confirmed that these primers amplified a putative pseudogene only (not shown). This primer pair was then used for the isolation of pseudogene-specific BAC clones by PCR screening (Incyte Genomics), which resulted in two positive clones, 398-H13 and 525-I20. The characterization of these BAC clones by nucleotide sequencing revealed that they both contain an ABCC6 pseudogene, ABCC6-ψ1, which is homologous to a region spanning exon 1 through intron 9 of the ABCC6 gene and its 5'- flanking sequences (Fig. 2). They also contain stop-codon-causing mutations 196insT in exon 2 and Q378X in exon 9. The nucleotide sequence of ABCC6-ψ1 contains a number of differences in the 5'-flanking region, and in exonic and intronic sequences, when compared with the functional ABCC6 gene (Table 1). In addition, one end of the clone 398-H13 shows homology to intron 18 of the PM5 gene, and further examination has revealed the pres-

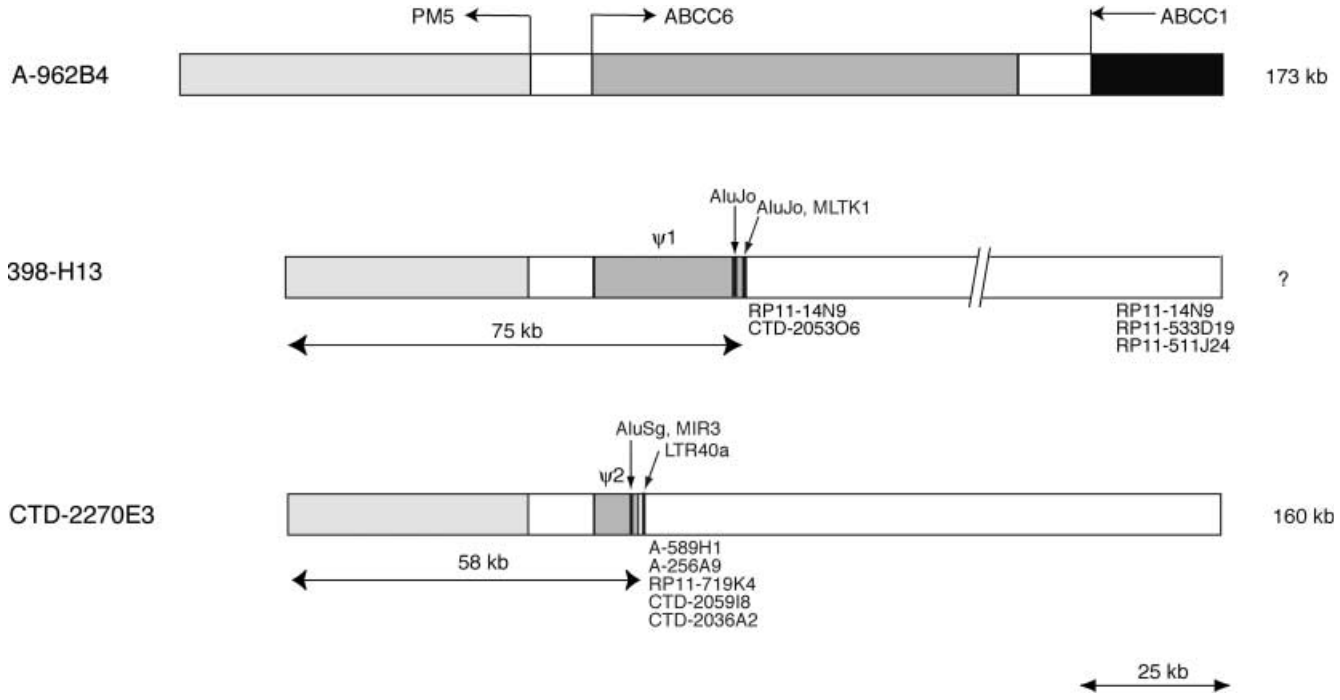

Fig. 2 Schematic presentation of BAC clones containing ABCC6 and its pseudogenes. The 173-kb BAC clone A-962B4 contains the full-length ABCC6 gene in a tail-to-tail orientation with the 3'-end of the ABCC1 gene and head-to-head orientation with the PM5 gene. The BAC clone 398-H13 contains ~25 kb of sequences representing ABCC6-ψ1 and ~41 kb corresponding to PM5 sequences from exon 1 to intron 18. These genes are part of a duplicon of ≥75 kb. The duplication breakpoint is surrounded by AluJo and retrovirus-like (MLTK1) repeat elements, and the region directly downstream has homology to BAC clones RP11–14N9 and CTD-205306. The other end of the 398-H13 clone has sequence homology to BAC clones RP11–14N9, RP11–533D19, and RP11–511J24. The 160-kb BAC clone CTD-2270E3 contains ~8.4 kb representing ABCC6-ψ2 and ~41 kb of PM5 spanning exon 1 to intron 18, which together with the intervening sequences form a duplicon of ≥58 kb. Directly downstream from the end of ABCC6-ψ2, a region with homology to a number of BAC clones (as indicated) was noted. The breakpoint of the duplicon is surrounded by AluSg and MIR3 repetitive elements, and endogenous retroviral-like elements (LTR40a). Although present also elsewhere, repetitive elements are indicated only in the vicinity of the duplication breakpoints

ence of a segment corresponding to exon 1 through intron 18 of this gene (Fig. 2). The region in clone 398-H13, which extends from intron 9 of ABCC6 upstream to intron 18 of PM5, spans ~75 kb when compared with the BAC clone A-962B4 (GeneBank accession no. U91318). The breakpoint of the duplicated region present in BAC clone 398-H13 within ABCC6-ψ1 resides within intron 9, 227 bp upstream from the 5'-end of exon 10. The very high homology between the coding regions of ABCC6-ψ1 and ABCC6 (99.995%) suggests that this duplication has occurred relatively recently. Sequencing of the BAC clone 398-H13 downstream from the ABCC6 breakpoint revealed an abundance of AluJo repeat sequences, and the sequence immediately beyond has homology to BAC clones RP11–14N9 and CTD-2053O6 (see Fig. 2). There is no sequence homology between ABCC6 and the sequences in these two BAC clones. The clone RP11–14N9 has been mapped ~0.28 Mb centromeric from the BAC clone A-962B4 (http//:genome.ucsc.edu). This map location for ABCC6-ψ1 was supported by marker content mapping, which demonstrated that the clones 398-H13 and 525-I20 are positive for marker D16S2799 (also known as s343D3), which maps to the same region (Cao et al. 1999). However, marker content mapping excluded 21 other markers examined (see above).

Evidence for a second pseudogene was obtained by the use of the BLAST search with ABCC6 specific sequences. The htgs database revealed sequences with high homology to ABCC6 in BAC clone CTD-2270E3 (GeneBank accession no. AC020757). Specifically, this clone has homology to the region spanning the 5'-flanking sequences and exon 1 through intron 4 segment. This ABCC6 copy was designated as pseudogene 2, ABCC6-ψ2. Some sequence differences in the 5'-flanking region and in exons 2, 3, and 4 were noted when ABCC6-ψ2 was compared with the corresponding region of ABCC6 (Table 1). The presence of these exonic nucleotide variations was also noted, during initial mutation screening, in a heterozygous state in all individuals examined, including PXE patients and normal unrelated healthy controls. Clone CTD-2770E3 also showed homology to PM5, one end of the clone starting upstream from intron 18 of this gene. The distance between the breakpoint in intron 4 of ABCC6 and the start site in intron 18 of PM5 is 58 kb, the same as the corresponding region in BAC clone A-962B4 (Fig. 2). This observation implies that there has not been any major genomic rearrangements during the duplication of this region, further suggesting that this duplication event is relatively recent. This conclusion is also supported by the high degree of homology between the coding regions of ABCC6-ψ2 and ABCC6 (99.996%). Based on the human genome work-

Table 2 ABCC6-specific primers designed on the basis of sequence differences between ABCC6 and its two pseudogenes. *Underlined* nucleotides eliminate the amplification of both pseudogenes, *underlined* nucleotides in *bold* (E3/4F) eliminate ABCC6-ψ2, *underlined* nucleotides in *italics* eliminate ABCC6-ψ1

Forward primer (5'–3' orientation)		Reverse primer (5'–3' orientation)		Size of amplimer (bp)	Annealing temperature (°C)
E1F	TGCTGGGTCCAAAGTGTTTA	E1R	CAGCCCGAGAGATCTGCAGC	469	55
E2F	GATCCAAAAAGTTGCCTGGC	E2R	TGTCCCCTGCCTCCCCCGAA	328	60
E3/4F	TCCCAGTTGGACATGGGGC**C**	E3/4R	TATAAGTGTGTGCATCGTG*T*	736	60
E5F	CCTCTGTCTCCATTCCTTA*T*	E5R	AGACTGAGACCTCAAAGTGG	219	55
E6F	CACAGTTCGTCCTGTCTTCC	E6R	GGCCCTGGAGAAGCAGCTG*T*	624	57
E7F	GATCCTGCAGGGGTGAATGG	E7R	ATGATGAGCTTTTCTGAAG*T*	242	50
E8F	CCCCCAACTCCCATGATTG*C*	E8R	AAGGATGCCACTAAGAGACC	450	55
E9F	AGGCACCTCCTCTCACCAGC	E9R	GGTGACAGAGCAAGACTCC*A*	423	60

ing draft database (http//:genome.ucsc.edu), BAC clone CTD-2270E3 maps ~1 Mb telomeric from ABCC6.

Based on the data presented above, we conclude that, in addition to the 20-kb to 45-kb duplicons described by Loftus et al. (1999), chromosome 16p also contains larger duplicons of at least 58–75 kb, which contain truncated versions of the ABCC6 and PM5 genes (Fig. 2).

Expression and transcriptional processing of the pseudogene ABCC6-ψ1

We subsequently studied the expression of the pseudogenes by determining the presence of ABCC6-ψ1 mRNA transcripts by utilizing reverse transcription/PCR (RT-PCR). The nested PCR with primer pairs cE1-F/cE9-R (first set) and cE1.1-F/cE8-R (second set) spanning the region from exon 1 to exon 8 and with keratinocyte or fibroblast mRNA as the template revealed the amplification of ABCC6-ψ1 sequences together with ABCC6. Sequence analysis demonstrated that ABCC6-ψ1 underwent transcriptional processing similar to that of the ABCC6 gene. However, in addition to normal splice products, analysis of individual clones after subcloning of the PCR products revealed an alternatively spliced variant. Specifically, a product containing 116 bp of intron 2 sequences was noted, when mRNA obtained from HEP-2 cells was used as the template with primers spanning exon 1 to exon 7 (cE1-F and cE7-R). The exon-intron borders of this alternative exon demonstrated consensus 3'-acceptor and 5'-donor splice sites, ag-EXON-gt.

Identification of novel mutations in ABCC6 by the use of allele-specific primers

Based on sequence differences between the ABCC6 gene and its pseudogenes, ABCC6-specific primers were designed for the amplification of each ABCC6 exon for mutation detection in patients with PXE (Table 2). The absence of pseudogene-specific nucleotide variants was verified by direct nucleotide sequencing of each PCR product. Subsequently, these primers were used for mutation detection by PCR amplification, followed by CSGE analysis. The PCR product spanning exon 2 demonstrated a heteroduplex band on CSGE, when DNA obtained from the proband of Family 1 with PXE was examined, whereas a homoduplex band in control samples was seen (Fig. 3A). Sequence analysis of the PCR product from the proband revealed a 9-bp deletion, 179del9, in a heterozygous stage (Fig. 3B, top). Sequencing of the corresponding PCR product from a normal control demonstrated the absence of pseudogene-specific nucleotide changes, 191G→A found in ABCC6-ψ2 and 196insT in ABCC6-ψ1 (Fig. 3B, bottom; compare with the sequence seen in Fig. 1). These data indicate that these primers are able not only to exclude pseudogene-specific sequences from the PCR product, but also to amplify both ABCC6 alleles. In his other allele, the proband in Family 1 was shown to harbor a G→C transversion at nucleotide position 4060 in exon 29, which resulted in the substitution of a glycine residue by arginine at amino acid position 1354 (G1354R; Fig. 3C). Thus, the proband was a compound heterozygote for ABCC6 mutations 179del9/G1354R (Fig. 3D). The mutations 179del9 and G1354R abolish *Nci*I and *Bst*NI restriction enzyme sites, respectively, which were used to verify the segregation of the mutations in the family (Fig. 3D), and their exclusion from 100 control individuals (not shown).

The PCR product from the proband in Family 2 showed a heteroduplex band, when the product spanning exon 9 was examined on CSGE (Fig. 4A). Direct sequencing of the PCR product revealed a nucleotide change 1091C→G, which resulted in the substitution of threonine by arginine (T364R; Fig. 4B). At the same time, the three pseudogene-specific nucleotide changes were absent from the PCR product (Fig. 4B; compare with Fig. 1). In addition to the missense mutation T364R, this patient was shown to have a recurrent nonsense mutation R1141X in exon 24 in the other ABCC6 allele, as shown by sequence analysis (Fig. 4C). Verification of mutation T364R was performed by mismatch primers, which created a *Sty*I restriction enzyme site in the mutant allele (see above). The presence of this mutation was excluded in 104 healthy control individuals by restriction enzyme digestion (not shown).

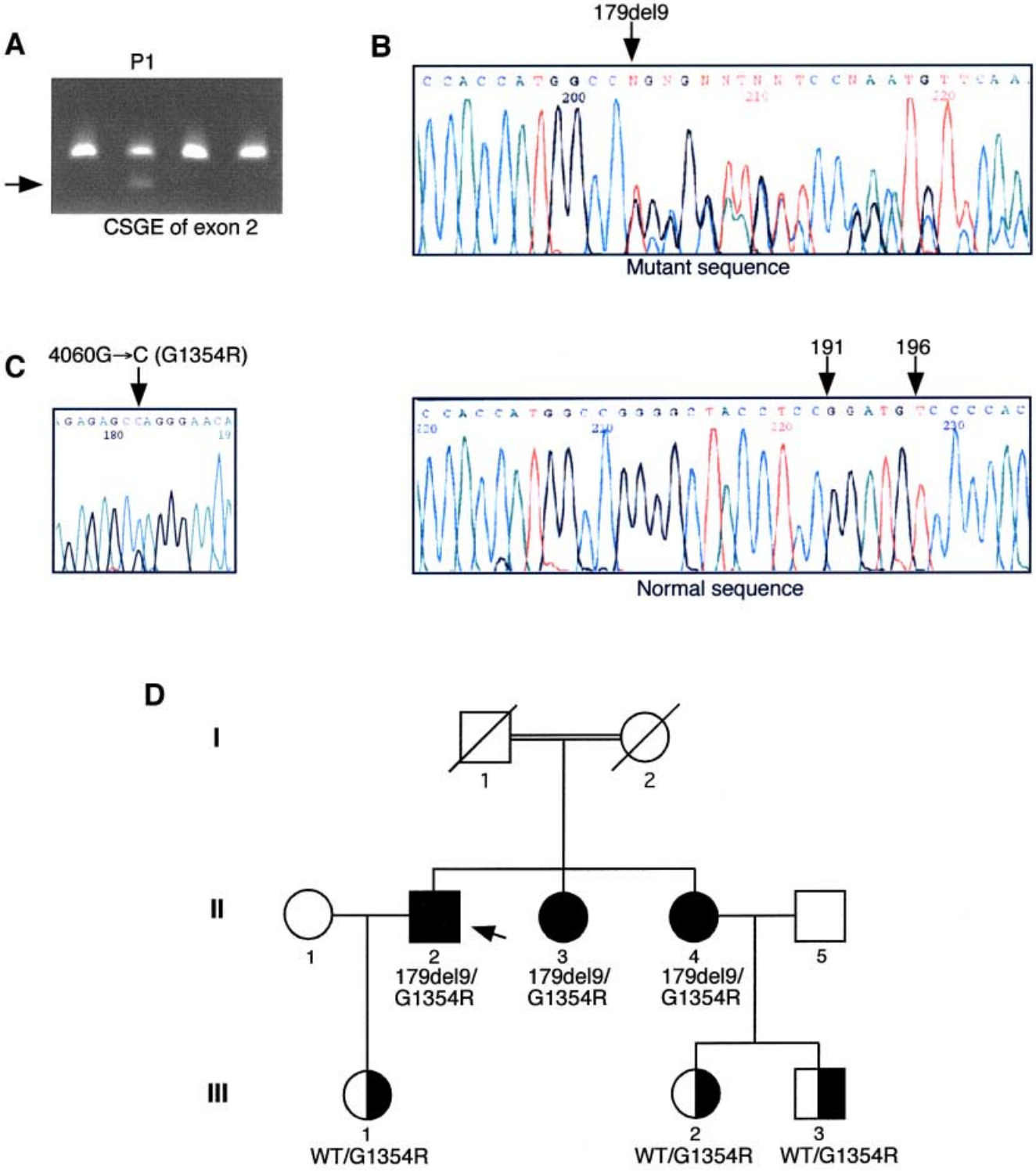

Fig. 3A–D Mutation analysis of the proband (*P1*) in Family 1 with PXE. ABCC6-specific primers were used for amplification of exons 1–9, and the PCR products were subjected to heteroduplex scanning by CSGE (**A**). A heteroduplex band (arrow) was noted when the PCR product spanning exon 2 was examined by CSGE. All other samples studied at the same time showed homoduplex bands only. Sequence analysis revealed a 9-bp deletion at nucleotide position 179 (**B**, *top*), compared with the control sequence (*bottom*), which was devoid of both ABCC6-ψ1 and ABCC6-ψ2 specific nucleotides (positions 191 and 196, compare with Fig. 1). The heteroduplex and sequence analyses of other regions of the ABCC6 gene revealed a G→C nucleotide transversion at position 4060 in exon 29; this resulted in substitution of a glycine residue by an arginine, a mutation designated as G1354R (**C**). Thus, the proband is a compound heterozygote for mutations 179del9/G1354R. The segregation of these mutations in the family is shown in **D**

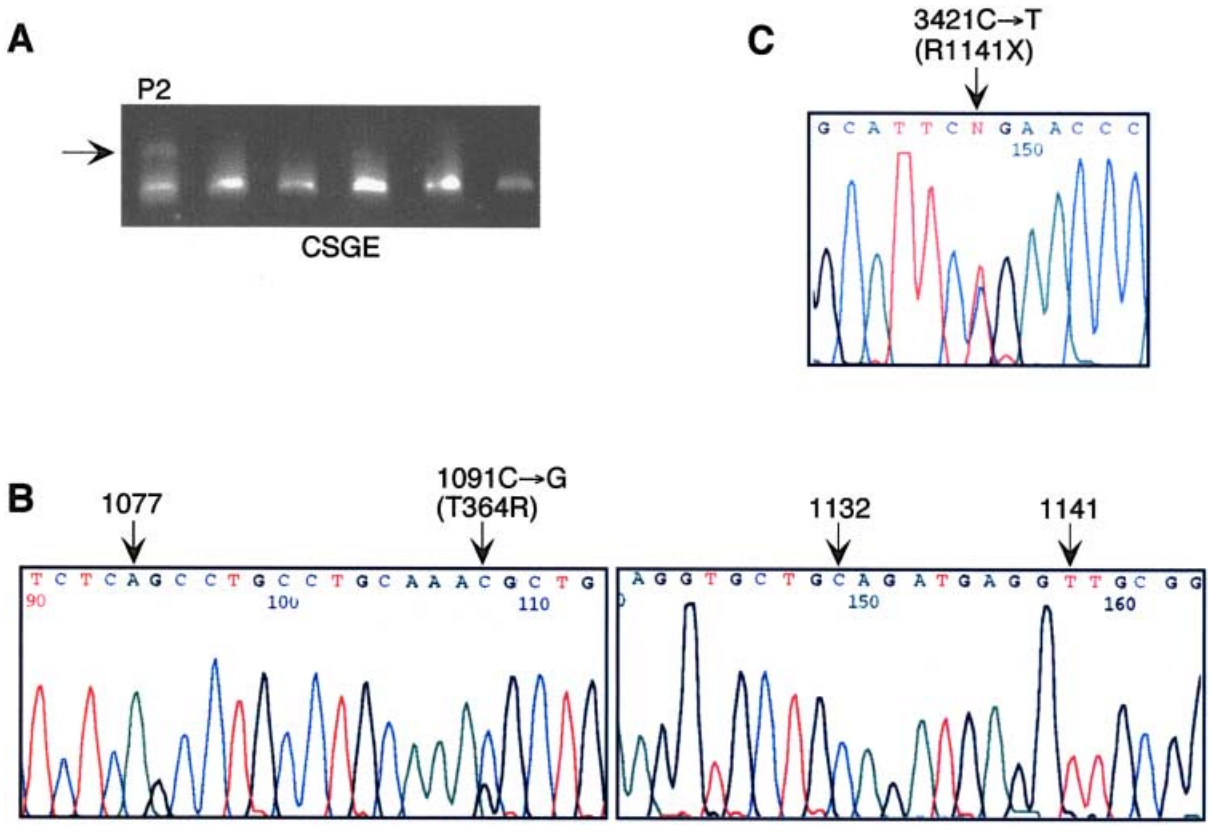

Fig. 4A–C Mutation analysis of the proband (*P2*) in Family 2 with PXE. CSGE analysis of PCR products spanning exon 9 revealed a heteroduplex band in the patient's sample (*arrow*), whereas all other PCR products showed homoduplex bands only (**A**). Nucleotide sequencing of the corresponding PCR product revealed a C→G transversion at position 1091, which results in the substitution of a threonine by an arginine residue at amino acid position 364 (T364R; **B**). Sequencing of this region revealed the absence of ABCC6-ψ1-specific nucleotides at positions 1077, 1132, and 1141 (**B**, compare with Fig. 1). Screening of other parts of the gene revealed a recurrent nonsense mutation R1141X in exon 24 in the other allele of patient P2 (**C**). Thus, the patient is a compound heterozygote for mutations T364R/R1141X

During the search for pathogenic mutations in ABCC6 in patients with PXE, we have also encountered several polymorphisms in the region spanning exons 1–9, by using allele-specific PCR (Table 3).

Discussion

Chromosome 16 is of considerable interest to molecular geneticists, as several disease genes, including those for PKD (Reeders et al. 1985), Batten disease (CLN3; Gardiner et al. 1990), and PXE (ABCC6; Le Saux et al. 1999; Cai et al. 2000) reside on this chromosome. Chromosome 16 has a particularly complex structure because it contains

Table 3 Polymorphisms characterized in the region spanning 5'-UTR and exon 1 to intron 9 of ABCC6, as identified by the use of allele-specific primers

Location	Nucleotide position[a]	Major allele	Minor allele	Allele frequences
5'-flanking	1–219	A	C	0.950/0.050
5'-flanking	1–132	C	T	0.953/0.047
5'-flanking	1–127	C	T	0.857/0.143
Exon 3	232	G (Ala)	T (Thr)	–
Intron 3	346–6	G	A	–
Exon 6	645	G (Thr)	A (Thr)	–
Intron 6	662+12	C	T	–
Intron 6	662+114	T	C	0.937/0.063
Intron 6	662+298	C	G	–
Intron 6	662+305	T	G	–
Intron 6	662+403	T	A	–
Exon 7	793	A (Arg)	G (Gly)	0.914/0.086

[a]The nucleotide positions refer to numbers in ABCC6 cDNA and are counted from the first nucleotide of the translation initiation codon ATG (Belinsky and Kruh 1999; GenBank accession nos. XM_007798, NM_001171)

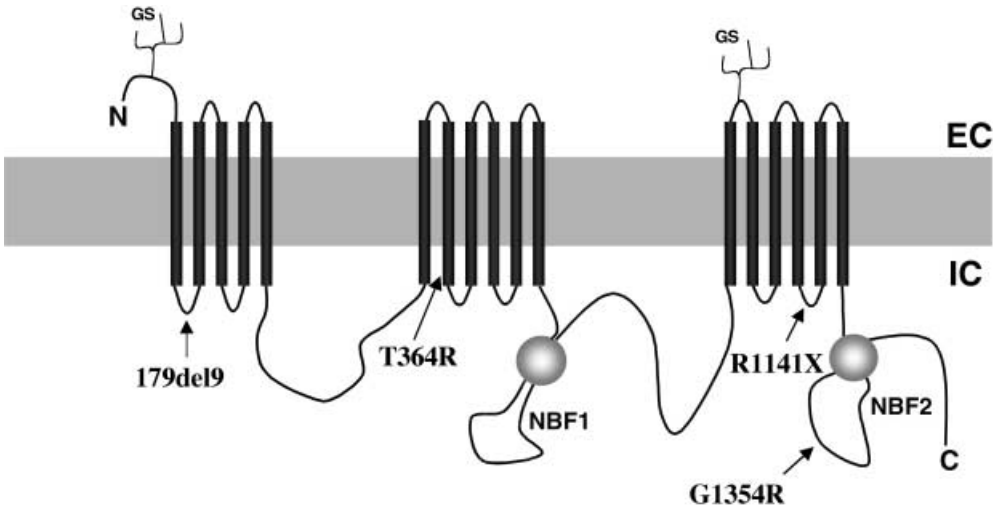

Fig. 5 Schematic presentation of MRP6, a multipass transmembrane protein. Note the presence of three transmembrane domains consisting of 5, 6, and 6 membrane-spanning segments (*vertical blocks*) and two nucleotide-binding folds (*NBF1*, *NBF2*). The positions of the mutations disclosed in the families with PXE are indicated by *arrows* (*EC* extracellular, *IC* intracellular, *GS* putative glycosylation sites)

a number of duplicated regions (Doggett et al. 1995; Loftus et al. 1999; Cai et al. 2000). This feature has caused difficulties in the characterization of the molecular basis of various inherited diseases, as illustrated previously by PKD (Watnick et al. 1997; Rossetti et al. 2001) and, in this study, by PXE. Loftus et al. (1999) have reported the presence of 20-kb to 45-kb duplicated regions (duplicons) that lie on chromosome 16, and that contain portions of known genes, such as PKD, or the entire coding region of eIF3-p110 gene. Here, we report, for the first time, the presence of two duplicated regions on chromosome 16p containing sequences with homology to ABCC6. Specifically, these duplicons are composed of truncated pseudogenes of ABCC6, viz., ABCC6-ψ1 and ABCC6-ψ2, and of the PM5 gene. These duplicons are at least 75 kb and 58 kb in size, or even larger, since it is unclear how far they extend beyond intron 18 of PM5. The high degree of homology between ABCC6 and its pseudogenes suggests, however, that these duplications have occurred relatively recently.

ABCC6-ψ1 was shown to be transcribed, as demonstrated by the presence of the corresponding mRNA in human epidermal keratinocytes, dermal fibroblasts, and HEP-2 cells. The mRNA was detectable by RT-PCR in spite of the presence of stop-codon mutations in exons 2 and 9, codons that could be thought to result in the down-regulation of the transcripts through nonsense-mediated mRNA decay (Henze and Kulozik 1999). The expression of this pseudogene from the duplicon could be explained by the presence of the promoter region with high degree of homology to that of ABCC6. Thus, the expression of this pseudogene should be taken into account when performing mutation analysis by RT-PCR. It should be noted that the mRNA levels of ABCC6 are not detectable in any of these cell types by Northern analysis (unpublished observations), suggesting that both ABCC6 and its pseudogenes are expressed at a low level in these cells.

The characterization of ABCC6 pseudogenes has implications for the molecular diagnostics of PXE, as exemplified by the two patients examined in this work. The use of ABCC6-specific primers eliminates the amplification of both pseudogenes in PCR products and, at the same time, allows reliable amplification of both ABCC6 alleles from genomic DNA. Furthermore, the identification of intragenic polymorphisms is valuable for the haplotyping of PXE families, if necessary. Specifically, haplotype analysis is useful for the detection of deletions and other genomic rearrangements in the ABCC6 gene. The ABCC6 locus is predicted to be prone to rearrangements, since this region is rich in Alu-repeats and other interspersed repetitive elements (Ringpfeil et al. 2001b). Indeed, we have previously reported a 16.5-kb intragenic deletion in ABCC6, which is apparently caused by Alu-mediated recombination (Ringpfeil et al. 2001b). The demonstration of Alu and retrovirus-like repetitive sequences adjacent to the breakpoints in the ABCC6 pseudogenes in the BAC clones characterized in this study (see Fig. 2) emphasizes that the repetitive elements may have played a role in the duplication of these sequences.

In this work, we have found three novel mutations, 179del9, T364R, and G1354R, and a recurrent nonsense mutation R1141X in two families with PXE (Fig. 5). In the proband of Family 1, the mutation 179del9 resides within the first transmembrane domain, specifically between the the first and second membrane-spanning segments, and causes deletion of arginine, glycine, and tyrosine residues from the MRP6 polypeptide (Fig. 5). Since this linking intracellular peptide is only 16 amino acids long, one could speculate that the shortening of the polypeptide by three amino acids interferes with its transmembrane topography. The second mutation *in trans* in the same patient is G1354R, which affects a conserved glycine residue in the second nucleotide-binding fold, a domain critical for the MRP6 protein to function as a transporter molecule. The proband in Family 2 is a compound heterozygote for R1141X and T364R mutations. The recurrent R1141X nonsense mutation predicts the elimination of the entire C-terminus from the protein, including part of the third transmembrane domain and the second nucleotide-binding fold (Fig. 5). The mutation T364R affects a conserved amino acid residue located within the second transmem-

brane domain and changes the amino acid charge, thus potentially altering the conformation and consequently the function of the protein. Therefore, all these mutations are located in regions that are critical for the function of MRP6 and are predicted to have pathogenetic consequences. Finally, it should be noted that heterozygous carriers of the mutations in Family 1 are clinically unaffected, thus confirming the recessive nature of these mutations.

In summary, we have identified two ABCC6 pseudogenes corresponding to the 5'-end of the gene on human chromosome 16p. The characterization of these pseudogenes has allowed us to design ABCC6-specific PCR primers that amplify exon 1–9 sequences of the ABCC6 gene only and that exclude the pseudogene sequences. These strategies facilitate the comprehensive analysis of ABCC6 for mutations in families with PXE.

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References

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402

Belinsky MG, Kruh GD (1999) MOAT-E (ARA) is a full-length MRP/cMOAT subfamily transporter expressed in kidney and liver. Br J Cancer 80:1342–1349

Bergen AAB, Plomp AS, Schuurman EJ, Terry S, Breuning M, Dauwerse H, Swart J, Kool M, Soest S van, Baas F, Brink JB ten, Jong PTVM de (2000) Mutations in ABCC6 cause pseudoxanthoma elasticum. Nat Genet 25:228–231

Borst P, Evers R, Kool M, Wijnholds J (1999) The multidrug resistance protein family. Biochim Biophys Acta 1461:347–357

Cai L, Struk B, Adams MD, Ji W, Haaf T, Kang HL, Dho SH, Xu X, Ringpfeil F, Nancarrow J, Zach S, Schaen L, Stumm M, Niu T, Chung J, Lunze K, Verrecchia B, Goldsmith LA, Viljoen D, Figuera LE, Fuchs W, Lebwohl M, Uitto J, Richards R, Hohl D, Ramesar R (2000) A 500-kb region on chromosome 16p13.1 contains the pseudoxanthoma elasticum locus: high-resolution mapping and genomic structure. J Mol Med 78:36–46

Cao Y, Kang HL, Xu X, Wang M, Dho SH, Huh JR, Lee B-J, Kalush F, Bocskai D, Ding Y, Tesmer JG, Lee J, Moon E, Jurecic V, Baldini A, Weier H-U, Doggett NA, Simon MI, Adams MD, Kim U-J (1999) A 12-Mb complete coverage BAC contig map in human chromosome 16p13.1-p11.2. Genome Res 9:763–774

Doggett NA, Goodwin LA, Tesmer JG, Meincke LJ, Bruce DC, Clark LM, Altherr MR, Ford AA, Chi HC, Marrone BL (1995) An integrated physical map of human chromosome 16. Nature 377(Suppl):335–365

Ganguly A, Rock MJ, Prockop DJ (1993) Conformation-sensitive gel electrophoresis for rapid detection of single-base differences in double-stranded PCR products and DNA fragments: evidence for solvent-induced bends in DNA heteroduplexes. Proc Natl Acad Sci USA 90:10325–10329

Gardiner M, Sandford A, Deadman M, Poulton J, Cookson W, Reeders S, Jokiaho I, Peltonen L, Eiberg H, Julier C (1990) Batten disease (Spielmeyer-Vogt disease, juvenile onset neuronal ceroid-lipofuscinosis) gene (CLN3) maps to human chromosome 16. Genomics 8:387–390

Hentze MW, Kulozik AE (1999) A perfect message: RNA surveillance and nonsense-mediated decay. Cell 96:307–310

Körkkö J, Annunen S, Pihlajamaa T, Prockop DJ, Ala-Kokko L (1998) Conformation sensitive gel electrophoresis for simple and accurate detection of mutations: comparison with denaturing gradient gel electrophoresis and nucleotide sequencing. Proc Natl Acad Sci USA 95:1681–1685

Le Saux O, Urban Z, Goring HH, Csiszar K, Pope FM, Richards A, Pasquali-Ronchetti I, Terry S, Bercovitch L, Lebwohl MG, Breuning M, Berg P van den, Kornet L, Doggett N, Ott J, Jong PTVM de, Bergen AAB, 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, Paepe A de, Boyd CD (2000) Mutations in a gene encoding an ABC transporter cause pseudoxanthoma elasticum. Nat Genet 25:223–227

Loftus BJ, Kim U-J, Sneddon VP, Kalush F, Brandon R, Fuhrmann J, Mason T, Crosby M, Barnstead M, Cronin L, Deslattes Mays A, Cao Y, Xu RX, Kang H-L, Mitchell S, Eichler EE, Harris PC, Venter JC, Adams MD (1999) Genome duplications and other features in 12 Mb of DNA sequence from human chromosome 16p and 16q. Genomics 60:295–308

Neldner K (1988) Pseudoxanthoma elasticum. Clin Dermatol 6: 1–159

Reeders ST, Breuning MH, Davies KE, Nicholls RD, Jarman AP, Higgs DR, Pearson PL, Weatherall DJ (1985) A highly polymorphic DNA marker linked to adult polycystic kidney disease on chromosome 16. Nature 317:542–544

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 USA 97:6001–6006

Ringpfeil F, Pulkkinen L, Uitto J (2001a) Molecular genetics of pseudoxanthoma elasticum. Exp Dermat 10:221–228

Ringpfeil F, Nakano A, Uitto J, Pulkkinen L (2001b) Compound heterozygosity for a recurrent 16.5-kb Alu-mediated deletion mutation and single-base-pair substitutions in the ABCC6 gene results in pseudoxanthoma elasticum. Am J Hum Genet 68:642–652

Rossetti S, Strmecki L, Gamble V, Burton S, Sneddon V, Peral B, Roy S, Bakkaloglu A, Komel R, Winearls CG, Harris PC (2001) Mutation analysis of the entire PKD1 gene: genetic and diagnostic implications. Am J Hum Genet 68:46–63

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Struk B, Cai L, Zach S, Ji W, Chung J, Lumsden A, Stumm M, Huber M, Schaen L, Kim CA, Goldsmith LA, Viljoen D, Figuera LE, Fuchs W, Munier F, Ramesar R, Hohl D, Richards R, Neldner KH, Lindpaintner K (2000) Mutations of the gene encoding the transmembrane transporter protein ABC-C6 cause pseudoxanthoma elasticum. J Mol Med 78:282–286

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

Watnick TJ, Piontek KB, Cordal TM, Weber H, Gandolph MA, Qian F, Lens XM, HPH Neumann, Germino GG (1997) An unusual pattern of mutations in the duplicated portion of *PKD1* is revealed by use of a novel strategy for mutation detection. Hum Mol Genet 6:1473–1481