Development of a Rapid, Reliable Genetic Test for Pseudoxanthoma Elasticum

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Mutations in the human *ABCC6* gene cause pseudoxanthoma elasticum (PXE), a hereditary disorder that impacts the skin, eyes, and cardiovascular system. Currently, the diagnosis of PXE is based on physical findings and histological examination of a biopsy of affected skin. We have combined two simple, polymerase chain reaction (PCR)-based methods to develop a rapid, reliable genetic assay for the majority of known PXE mutations. After PCR amplification and heteroduplex formation, mutations in exon 24 and exon 28 of the *ABCC6* gene were detected with Surveyor nuclease, which cleaves double-stranded DNA at any mismatch site. Mutations originating from deletion of a segment of the *ABCC6* gene between exon 23 and exon 29 (ex23_ex29del) were detected by long-range PCR. Size analysis of digestion fragments and long-range PCR products was performed by agarose gel electrophoresis. The methods accurately identified mutations or the absence thereof in 16 affected individuals as confirmed by DNA sequencing. Fifteen patients had one or two point mutations, and two of these individuals carried the ex23_ex29del in their second allele. This mutation detection and mapping strategy provides a simple and reliable genetic assay to assist in diagnosis of PXE, differential diagnosis of PXE-like conditions, and study of PXE genetics. (J Mol Diagn 2007, 9:105–112; DOI: 10.2353/jmoldx.2007.060093)

Pseudoxanthoma elasticum (PXE) is a human hereditary disorder of the *ABCC6* gene (Online Mendelian Inheritance of Man no. 603234) that involves primarily the skin and eye, as well as occasionally the gastrointestinal and cardiovascular systems (Online Mendelian Inheritance of Man no. 264800). The characteristic clinical manifestations are the presence of yellowish papules and plaques leading to laxity and redundancy in flexural areas and angioid streaks in Bruch's membrane behind the retina, which is associated with choroidal neovascularization, hemorrhage, and subsequent central vision loss. Currently, diagnosis of PXE relies on clinical examination for

characteristic skin lesions and angioid streaks or von Kossa staining of a biopsy of lesional skin looking for calcification of dystrophic dermal elastic fibers.¹ However, high individual variability in severity, phenotype, and disease onset and progression can complicate the diagnosis, even among affected siblings with identical mutations.² There is a need for a definitive tool for diagnosis, particularly for siblings of affected individuals.

The *ABCC6* gene (Online Mendelian Inheritance of Man no. 603234) consists of 31 exons on human chromosome 16p13.1.^{3–6} The gene encodes a protein (ABCC6/MRP6) belonging to the ATP-binding cassette membrane transporter family with 1503 amino acid residues, three transmembrane segments consisting of 17 hydrophobic helices, and two conserved nucleotide binding domains (NBD1 and NBD2).^{7–9} *ABCC6* gene mutations have been associated with autosomal recessive and sporadic forms of PXE.^{5,10–13} At present, some 150 causative mutations in this gene have been observed in different populations, with most mutations being missense, nonsense, deletion/insertion, or splice site alterations clustered toward the large carboxyl-terminal end of ABCC6/MRP6 in NBD1 and NBD2.^{5,10–30} The most frequent mutations in North American, European, and South African populations are c.3421C>T (p.R1141X) in exon 24 and Alu-mediated deletion of sequences between exon 23 and 29 (ex23_ex29del).^{14,16,18,19,21,23} Mutations in the *ABCC6* gene that cause PXE allow development of genetic tests for accurate clinical diagnosis, differential diagnosis from PXE-like phenotypes (eg, PXE-like papillary dermal elastolysis and fibroelastolytic papulosis, periumbilical perforating PXE, PXE-like presentation of β -thalassemia, and acquired PXE syndromes), and predictive preclinical diagnosis to allow for possible intervention and for timely genetic counseling.

Surveyor nuclease is a member of the CEL DNA endonuclease family of enzymes that specifically cleaves mismatched base pairs in DNA heteroduplexes, including single-base substitutions, deletions, and insertions.^{31–33} The mismatch-cutting activity of CEL nuclease family members has been used in a number of different applications designed to detect genetic variations.^{34–38} Here, we applied this technology to PXE genetic diagnosis in detection of mutations in exon 24 and exon 28 of the

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ABCC6 gene. In addition, we used long-range polymerase chain reaction (PCR) to identify ex23_ex29del mutations in the *ABCC6* gene.¹⁶ Agarose gel electrophoresis was used to analyze nuclease digestion products and long-range PCR products. The purpose of this study is to show that the combined use of these methods provides a simple and reliable test to screen for the most common disease-causing mutations in the *ABCC6* gene.

Materials and Methods

Reagents

Primers were custom synthesized by Invitrogen (Carlsbad, CA). Optimase polymerase, 10× Optimase reaction buffer, dNTPs, Surveyor Nuclease S, and TransOneK agarose were supplied by Transgenomic, Inc. (Omaha, NE).

Patient Genomic DNA Samples

Coded patient genomic DNA samples were obtained from the PXE International/Genetic Alliance BioBank. PXE International is a not-for-profit foundation that initiates, conducts, and funds research on PXE. Donors were recruited by PXE International, underwent an informed decision-making process, and gave informed consent. The protocol was approved by the Genetic Alliance BioBank Institutional Review Board. Donors were considered positive for PXE if they met at least two of the following three conditions: skin biopsy demonstrating calcification of dystrophic elastic fibers in the dermis, the presence of angioid streaks in the retina, or positive family history of PXE.³⁹ Genomic DNA was isolated from whole blood (Puregene DNA isolation kit; Gentra Systems, Minneapolis, MN). Normal genomic DNA was obtained from the human cell line K562 (American Type Culture Collection, Manassas, VA). Coded samples were each randomly assigned numbers from 1 to 16 for identification purposes. Blinded analyses for nuclease assay and long-range PCR were performed on this DNA set.

ABCC6 Mutations Selected for Analysis

Studies of cohorts from North America, Europe, and other populations have identified ~150 disease-causing mutations in the *ABCC6* gene.^{5,10–30} Whereas neutral variants are evenly distributed, pathogenic mutations are clustered at the downstream end of the *ABCC6* gene, particularly in exon 24 and in exons 28 to 30. The c.3421C>T (p.R1141X) mutation in exon 24 and the deletion between exon 23 and exon 29 (ex23_ex29del) are those most commonly found in phenotypically positive samples.^{14,16,18,19,21,23} This published study and unpublished research sponsored by PXE International, in collaboration with Transgenomic, Inc.; Jefferson Medical College, Philadelphia, PA; Ghent University, Ghent, Belgium; and the University of Witwatersrand, Johannesburg, South Africa, determined that mutations in exons 24 and 28 and the deletion of exons 23 to 29 account for ~70% of the *ABCC6* gene mutations in individuals af-

ected by PXE. Mutation screening in this report was therefore targeted to *ABCC6* gene exon 24 and exon 28 using Surveyor nuclease to detect point mutations³³ and to the ex23_ex29del using long-range PCR.¹⁶ Analysis with the nuclease assay could easily be expanded to include detection of point mutations and small deletions and insertions in other regions of the *ABCC6* gene where mutations correlate to PXE.

Mutation Detection with the Nuclease Assay

Mutation detection with the nuclease assay involves four steps: 1) PCR amplification of patient genomic DNA; 2) hybridization of the PCR product to form heteroduplexes; 3) digestion of hybridized DNA with Surveyor nuclease; and 4) fragment analysis by agarose gel electrophoresis (Figure 1).

PCR Amplification of *ABCC6* Exon 24 and Exon 28

Amplified DNA, free of secondary products and primer-dimers, is essential for reliable nuclease mutation detection. Nested PCR was the only approach we found that produced single-product, quality DNA in high, uniform yield from *ABCC6* gene exon 24 and exon 28 sequences in PXE patient genomic DNA. The primers used to amplify exons 24 and 28 were designed to bind well outside the exon sequences themselves so that cleavage of the amplified DNA fragments at any mutation in exon 24 or exon 28 will produce fragments >100 bp long. Fragments of this length or longer bind sufficient ethidium bromide so that they can be visualized easily in agarose gels. Fragments substantially <100 bp in length, resulting from cleavage well outside exons 24 and 28, will not be seen in agarose gels.

The PCR primers used in the nested PCR protocol for preparing amplified DNA from PXE exon 24 and 28 are listed in Table 1. In the first round of nested PCR, reaction mixtures (50 µl) contained 1× Optimase reaction buffer, 0.2 mmol/L of each dNTP, 0.2 µmol/L each of outer forward and reverse primer, 2.5 U of Optimase polymerase, and 10 ng of genomic DNA. The PCR program was 95°C for 2 minutes; 14 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute; 20 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute; 72°C for 5 minutes; and hold at 4°C. Using the same protocol as above, the second round of nested PCR was performed in a reaction mixture (50 µl) containing identical components except that 0.5 µl of PCR product from the first PCR amplification was used as template and an inner primer pair replaced the outer primer pair (Table 1). An aliquot (5 µl) of the final PCR product was analyzed for its quality and concentration by 2% agarose gel electrophoresis in 1× TBE buffer (89 mmol/L Tris-borate, pH 8.3, 1 mmol/L ethylenediaminetetraacetic acid) with ethidium bromide staining.

Heteroduplex Formation

Before nuclease digestion, exon 24 and exon 28 PCR products were both self-hybridized and cross-hybridized with

Step 4: Fragment Analysis

Agarose Gel Electrophoresis

Figure 1. Nuclease mutation detection involves four steps: 1) nested PCR amplification of *ABCC6* gene exons from the sample and normal reference genomic DNA; 2) self-hybridization of the sample DNA and cross-hybridization with normal reference DNA to form DNA heteroduplexes by heating and gradual cooling; 3) Surveyor nuclease digestion to cleave the mismatches in the heteroduplexes; and 4) mutation fragment analysis by agarose gel electrophoresis.

normal amplified DNA. Self-hybridization produces DNA heteroduplexes when the DNA sample is heterozygous for a mutation. Cross-hybridization with normal amplified DNA is required to produce heteroduplexes when the DNA sample is homozygous for a mutation or when the PCR target sequence has been deleted in one allele. Equal amounts of PCR-amplified normal DNA and patient DNA were mixed and hybridized directly in PCR reaction buffer without cleanup. Hybridization was performed in a PCR thermocycler using the following program: 95°C for 2 minutes; 95 to 85°C at a cooling rate of $-2^{\circ}\text{C}/\text{second}$; 85 to 25°C at a cooling rate of $-0.2^{\circ}\text{C}/\text{second}$; and hold at 4°C.

Nuclease Digestion

Hybridized DNA (5 μl) amplified from exon 24 or exon 28 sequences was digested with 1 μl of Surveyor Nuclease S directly in PCR reaction buffer at 42°C for 20 minutes. The incubation was terminated by addition of 1/10 vol of 0.5 mol/L ethylenediaminetetraacetic acid (pH 8.0).

DNA Fragment Analysis

DNA samples (5 μl) were analyzed by agarose [2.5% (w/v); Transgenomic, Inc.] gel electrophoresis in gels cast in 1 $\times$

Table 1. Primers Used for PCR Amplification of PXE Gene Sequences

Primer name	Region amplified	Nested PCR round	Primer sequence (5' to 3')	Nucleotide numbers*
24Fo	Exon 24	First	5'-GTGATTTTCAAAAAAACCATAGAACTCTGATTC-3'	43,039 to 43,007
24Ro	Exon 24	First	5'-CATGTTCCATCTGCCCACGGTGAGAAC-3'	42,470 to 42,496
24Fi	Exon 24	Second	5'-GCAAGGAAATGAGAACCCAGAGAGGGCAAG-3'	43,005 to 42,976
24Ri	Exon 24	Second	5'-GATAGACTGCCTGTGGGATCTAGCCTC-3'	42,498 to 42,524
28Fo	Exon 28	First	5'-GGAAGGTAGACCTTACACAATGAGGGATG-3'	34,798 to 34,769
28Ro	Exon 28	First	5'-CTTTGAGCTGCACCGTCTCCAGGGCTGC-3'	34,335 to 34,362
28Fi	Exon 28	Second	5'-GATAGACAGATCTCGGGTACAGCAGAAAG-3'	34,768 to 34,740
28Ri	Exon 28	Second	5'-CAGATAGCCTCGTCCGAGTGCTCCTGCAG-3'	34,364 to 34,392
dF1	Exon 23/29 Del	na [†]	5'-CAGTGGGAATCTCAAGGCGAATGGTTAC-3'	47,667 to 47,640
dF2	Exon 23/29 Del	na	5'-GATTCTTGAAGCAGCAGTGAGTCATTGTAG-3'	31,150 to 31,121
dR	Exon 23/29 Del	na	5'-CTTTGAGCAGGCTGACTGTAGGCAGGTC-3'	30,594 to 30,621

*Nucleotide numbering was taken from BAC clone A-962B4 (accession number U91318).¹⁶

[†]na, not applicable.

TBE buffer with 0.5 $\mu\text{g/ml}$ ethidium bromide and run in 1 $\times$ TBE. In some cases a Wide Mini ReadySub-Cell GT system with a precast TBE Wide Mini Ready agarose gel, 3% plus ethidium bromide (Bio-Rad, Hercules, CA) was used for gel analysis. A 100-bp DNA ladder (New England BioLabs, Beverly, MA) was run as a size marker. Digital images of the gel were taken under UV transillumination (250 nm).

Long-Range PCR Amplification for Detection of ABCC6 Exon 23/29 Deletions

To detect *ABCC6* ex23_ex29del mutations, long-range PCR was used.¹⁶ PCR was performed with three primers: dF1, dF2, and dR (Table 1). By using two forward primers, dF1 and dF2, one before and the other after the breakpoint, and a reverse primer, dR, one additional 672-bp DNA fragment of approximately equal intensity as the normal 577-bp fragment appears when the 16.5-kb deletion brings the dF1 and dR primers within the PCR DNA polymerase's amplification size range. PCR reaction mixtures (50 μl) contained 1 $\times$ Optimase reaction buffer, 0.2 mmol/L of each dNTP, 0.2 $\mu\text{mol/L}$ dF1 primer, 0.2 $\mu\text{mol/L}$ dF2 primer, 0.4 $\mu\text{mol/L}$ dR primer, 2.5 U of Optimase polymerase, and 10 ng of template DNA. The PCR program was the same as that used for exons 24 and 28. PCR products were analyzed directly by agarose gel electrophoresis.

DNA Sequencing

Nested PCR products were purified (Qiagen PCR Cleanup kit; Qiagen, Valencia, CA) and labeled using a 24Fi, 24Ri, 28Fi, or 28Ri primer and a BigDye Terminator DNA sequencing kit (Applied Biosystems, Foster City, CA). Sequencing was performed in an ABI Prism 3100 genetic analyzer (Applied Biosystems).

Results

Nuclease Mutation Detection in *ABCC6* Exon 24 and Exon 28

Table 2 lists the most common mutations in *ABCC6* gene exons 24 and 28 associated with PXE. Also listed are the lengths of the exon 24 and 28 amplicons and the sizes of the DNA fragments generated by nuclease cleavage at the mutation sites. PXE exon 24 and exon 28 DNA sequences were PCR amplified from 16 patient DNA samples and from normal reference DNA. After self-hybridization of the sample DNAs and cross-hybridization with reference DNA, the DNAs were digested with Surveyor nuclease, and the digestion mixtures were subjected to analysis by agarose gel electrophoresis (Figures 2 and 3). Faster migrating mutation cleavage fragments along with undigested homoduplex substrate DNA were readily separated and detected on agarose gels.

Single mutations were identified in exon 24 in 11 patient DNA samples (Figure 2A), including the DNA from patients 1 to 7, 11, and 13 to 15. The DNA from one patient (patient 12) had two mutations in exon 24, indi-

Table 2. Nuclease Digestion Fragment Sizes of Common Mutations in PXE Exon 24 and 28

Amino acid change	Base change	Fragment lengths (bp)
p.T1130M	c.3389C>T	251,257/508
p.R1138W	c.3412C>T	274,234/508
p.R1138Q	c.3413G>A	275,233/508
p.R1141X	c.3421C>T	281,227/508
p.R1164X	c.3490C>T	352,156/508
p.G1302R	c.3904G>A	116,289/405
p.R1314Q	c.3941G>A	153,252/405
p.R1339C	c.4015C>T	227,178/405

The total lengths of the amplicons are listed after the slash.

cated by the presence of four digestion fragments (Figure 2, A and B; lane 12). Self-hybridized DNA from patients 1, 5, 6, 11 to 13, and 15 produced nuclease digestion fragments, indicating these individuals had heterozygous *ABCC6* alleles (Figure 2B). Patients 2 to 4, 7, and 14 yielded no digestion fragments from self-hybridized DNAs but did give digestion fragments from cross-hybridized DNAs; therefore these individuals were either homozygous for the mutation(s) or had the exon 24 region deleted in one allele. The estimated fragment sizes of the exon 24 mutations in 12 of 12 samples matched the calculated sizes of known exon 24 allelic variants (Table 2), allowing us to make tentative mutation assignments from the list in Table 2 (Table 3). Because of the one base position difference between mutation c.3412C>T (p.R1138W) and c.3413G>A (p.R1138Q) in exon 24 and the 8- and 9-base position differences between the c.3413G>A and c.3412C>T mutations and the c.3421C>T (p.R1141X) mutation in exon 24, the cleavage fragments from these mutations could not be distinguished by agarose gel electrophoresis. The mutations identified in this region were therefore designated in Table 3 as c.3412C>T, c.3413G>A, or c.3421C>T.

Mutations were identified in exon 28 in the DNA of five patients, 4, 5, 8, 9, and 16 (Figure 3A), one of which (patient 8) was homozygous for the mutation or had the exon 28 region deleted in one allele (compare Figure 3, A and B; lane 8). Estimates of mutation cleavage fragment

Figure 2. Mutation detection in PXE exon 24 by nuclease digestion and agarose gel electrophoresis. Amplified, hybridized DNAs were digested with Surveyor nuclease, and digestion products were analyzed by agarose gel electrophoresis (see Materials and Methods). In **A**, the samples were cross-hybridized with normal DNA, and in **B**, the samples were self-hybridized. Patient samples coded 1 to 16 were run in correspondingly numbered lanes. Digested normal DNA was run in **lane 0**, and 100-bp DNA ladder in **lane M**. **Arrows** indicate mutation cleavage fragments.

Figure 3. Mutation detection in PXE exon 28 by nuclease digestion and agarose gel electrophoresis. Amplified, hybridized DNAs were digested with Surveyor nuclease, and digestion products were analyzed by agarose gel electrophoresis (see Materials and Methods). In **A**, the samples were cross-hybridized with normal DNA, and in **B**, the samples were self-hybridized. Patient samples coded 1 to 16 were run in the correspondingly numbered lanes. Digested normal DNA was run in **lane 0**, and 100-bp DNA ladder in **lane M**. **Arrows** indicate genetic variation cleavage fragments.

sizes allowed us to make tentative exon 28 mutation assignments to some of the mutations from the list in Table 2 (Table 4). Patients 5 and 16 each had a mutation corresponding to c.4015C>T (p.R1339C) (cleavage fragments of ~180 and ~230 bp), and patients 8 and 9 had one mutation corresponding to c.3904G>A (p.G1302R) (fragments of ~120 and ~290 bp). Digestion of DNA from patients 4, 5, and 16 also produced a pair of cleavage fragments ~100 and ~300 bp long whose positions of migration differed slightly from those of the c.3904G>A pair (Figure 3, A and B). This result was consistent with the presence of a SNP in the intronic region between exon 28 and exon 29. The exon 28 PCR product actually includes part of the intronic sequences between exons 27 and 28, all of exon 28, all of the intronic sequences between exons 28 and 29, as well as part of exon 29. Sample 10 had no mutations in either exon 24 or exon 28.

Sequence Verification of ABCC6 Exon 24 and Exon 28 Mutations

With appropriately placed PCR primers, Surveyor nuclease can be used to identify any point mutation, or the lack thereof, in *ABCC6* exon 24 and exon 28. We determined the DNA sequence of exon 24 and exon 28 in all 16 patient DNAs and in the reference DNA to verify that the mutation assignments made with the nuclease assay were correct. Standard Big Dye Terminator sequencing by ABI genetic analyzer with 24Fi, 24Ri, 28Fi, and 28Ri primers (Table 1) on purified PCR products generated good signals in both directions (data not shown). Examination of the traces confirmed that within the fragment size resolution limitations of agarose gel electrophoresis, all genetic variations were correctly identified by the nuclease assay (Tables 3 and 4). The SNP in the amplified DNA of patients 4, 5, and 16 (Table 4) was determined to be IVS28 + 49C>T.

Long-Range PCR Amplification of PXE DNA to Detect the Exon 23 to 29 Deletion Mutation

Because the exon 23 to exon 29 16.5-kb genomic deletion is relatively frequent in PXE, particularly in the North America population,^{13,16} we also included a test for ex23_ex29del mutations.¹⁶ Using primers dF1, dF2, and dR (Table 1), PCR amplification of normal DNA produces a 577-bp fragment, whereas amplification of DNA containing the heterozygous ex23_ex29del mutation produces the same fragment and an additional 672-bp fragment. All 16 patient DNAs were amplified with dF1, dF2, and dR, and the PCR products were analyzed for ex23_ex29 del mutations by agarose gel electrophoresis (Figure 4). Two patient DNA samples (patients 7 and 14) were identified as carrying the ex23_ex29del mutation.

Table 3. A Summary of the Point Mutations Assigned in Exon 24 in the DNA of 16 PXE Patients by Nuclease Digestion and Fragment Analysis and by DNA Sequencing

Sample number	Mutations assigned based upon analysis by	
	Nuclease digestion	DNA sequencing
0 (normal)	-/-	-/-
1	c.3490C>T (+/-)	c.3490C>T (+/-)
2	c.3490C>T (+/+)	c.3490C>T (+/+)
3	c.3412C>T (+/+), c.3413G>A (+/+) or c.3421C>T (+/+)	c.3421C>T (+/+)
4	c.3412C>T (+/+), c.3413G>A (+/+) or c.3421C>T (+/+)	c.3413G>A (+/+)
5	c.3412C>T (+/-), c.3413G>A (+/-) or c.3421C>T (+/-)	c.3421C>T (+/-)
6	c.3412C>T (+/-), c.3413G>A (+/-) or c.3421C>T (+/-)	c.3421C>T (+/-)
7	c.3490C>T (+/+)	c.3490C>T (+/+)
8	-/-	-/-
9	-/-	-/-
10	-/-	-/-
11	c.3412C>T (+/-), c.3413G>A (+/-) or c.3421C>T (+/-)	c.3412C>T (+/-)
12	c.3412C>T (+/-), c.3413G>A (+/-) or c.3421C>T (+/-) and c.3490C>T (+/-)	c.3421C>T (+/-) and c.3490C>T (+/-)
13	c.3412C>T (+/-), c.3413G>A (+/-) or c.3421C>T (+/-)	c.3413G>A (+/-)
14	c.3389C>T (+/+)	c.3389C>T (+/+)
15	c.3412C>T (+/-), c.3413G>A (+/-) or c.3421C>T (+/-)	c.3412C>T (+/-) and c.3421C>T (+/-)
16	-/-	-/-

The mutant allele is designated (+), and a normal allele is (-). Mutants designated (+/+) could either have the same point mutation in both alleles or have the designated point mutation in one allele and a deletion of the sequence of interest in the other allele.

Table 4. A Summary of the Genetic Variations Assigned in Exon 28 in the DNA of 16 PXE Patients by Nuclease Digestion and Fragment Analysis and by DNA Sequencing

Sample number	Variations assigned based upon analysis by	
	Nuclease digestion	DNA sequencing
0 (normal)	-/-	-/-
1	-/-	-/-
2	-/-	-/-
3	-/-	-/-
4	SNP (+/-)	IVS28 + 49C>T (+/-)
5	c.4015C>T (+/-) and SNP (+/-)	c.4015C>T (+/-) and IVS28 + 49C>T (+/-)
6	-/-	-/-
7	-/-	-/-
8	c.3904G>A (+/+)	c.3904G>A (+/+)
9	c.3904G>A (+/-)	c.3904G>A (+/-)
10	-/-	-/-
11	-/-	-/-
12	-/-	-/-
13	-/-	-/-
14	-/-	-/-
15	-/-	-/-
16	c.4015C>T (+/-) and SNP (+/-)	c.4015C>T (+/-) and IVS28 + 49C>T (+/-)

The mutant allele is designated (+), and a normal allele is (-). Variants designated (+/+) could either have the same point mutation in both alleles or have the designated point mutation in one allele and a deletion of the sequence of interest in the other allele.

Summary of the Genetic Variations Found

The methods used in this test were reproducible. The nuclease assay and long-range PCR analyses of the 16 patient samples were repeated twice with identical results. DNA sequencing and nuclease digestion analysis were in agreement within the limitations of estimating fragment sizes by agarose gel electrophoresis (Tables 3 and 4), so that no false-negative or false-positive variations were observed. The one patient DNA, patient 10, that lacked mutations in the regions tested was found to have the mutation c.2836C>A (p.L946I) in exon 22 by denaturing high-performance liquid chromatography and DNA sequencing (data not shown). Table 5 summarizes the frequency of the genetic variations found in the *ABCC6* gene regions examined in the 16 PXE patient DNAs. The two most prevalent mutations were the nonsense mutations c.3421C>T (p.R1141X) and c.3490C>T (p.R1164X) in exon 24. c.3421C>T is the most common mutation found in PXE patients of European origin.^{14,19,20,23,29} c.3490C>T is a common mutation in individuals of British descent.^{16,21} Two DNA samples carried the c.3490C>T (p.R1339C) missense mutation in one exon 28 allele, and in both cases, IVS28 + 49C>T was also present. In a much larger patient sampling, we have found c.3940C>T is linked to IVS28 + 49C>T (13 of 13 samples; unpublished data).

Figure 4. Detection of PXE ex23_ex29del mutations by PCR and agarose gel electrophoresis of amplified DNA fragments. Patient samples coded 1 to 16 were run in the correspondingly numbered lanes. Normal DNA is designated 0, and 100-bp DNA ladder is M. Arrows indicate samples having a PCR product generated from a deletion mutation.

Discussion

Because the link between mutations in the carboxyl end of the human *ABCC6*/MRP6 protein and PXE has been established, it is important to develop methods that allow routine, reliable detection of these mutations. We have developed a simple strategy for detecting the most common mutations in the *ABCC6* gene that cause PXE by combining the use of PCR amplification and where appropriate, Surveyor nuclease digestion of DNA, with DNA fragment analysis on agarose gels. This strategy, which has been incorporated into a genetic assay, has advantages for use in the research and clinical laboratory because it: 1) involves relatively low investment in equipment and instrumentation, requiring only a PCR thermocycler, an agarose gel electrophoresis apparatus, and a gel photography system; 2) is performed directly with PCR products without further DNA purification; 3) gives information about the location of a mutation making tentative mutation identification possible; and 4) produces clear readouts without the full trace visual inspection required in DNA sequencing. In the blinded study described here, the assay correctly identified the presence of all mutations in a cohort of 16 PXE patient samples with 100% accuracy. No false positives were identified. Further validation studies are being performed on DNA from a larger patient population using this assay.

Although Surveyor nuclease mutation detection provides base change location but not base change identification, it effectively screens out normal samples without requiring DNA sequence analysis. Because at present most PXE mutations have been documented, nuclease digestion fragment sizes are often sufficient to determine tentatively the identity of a mutation by referring to theoretical fragment sizes produced from known mutations. Identification of closely spaced mutations can be aided by the use of a high-resolution platform. For example, we have also analyzed nuclease digestion products of exon 24 and exon 28

Table 5. Summary of Genetic Variations Found in the *ABCC6* Gene of 16 PXE Patients

Type of variation	Amino acid change	Sequence variation	Location	Alleles	Allelic state*
Missense	p.T1130M	c.3389C>T	Exon 24	1	ch
	p.R1138W	c.3412C>T	Exon 24	2	ht, ch
	p.R1138Q	c.3413G>A	Exon 24	2	hm, ht, ch
	p.G1302R	c.3904G>A	Exon 28	2	hm, ht
	p.R1339C	c.4015C>T	Exon 28	2	ch
Non-sense	p.R1141X	c.3421C>T	Exon 24	5	hm, ht, ch
	p.R1164X	c.3490C>T	Exon 24	4	hm, ht, ch
Deletion	p.I1000_S1403del W1404fsX1463	c.2996_4208del	Exons 23–29	2	ch
SNP		IVS28 + 49C>T	Intron 28	3	ch

*hm, homozygote; ht, heterozygote; ch, compound heterozygote.

PCR amplified DNA from all 16 patients on an ABI Prism 3100 genetic analyzer. The 5' end of the substrate was labeled with fluorescein. The results confirmed and extended those obtained with agarose gels. In patients 3 to 6, 11 to 13, and 15, mutations c.3412C>T and c.3413G>A could be differentiated from c.3421C>T. Direct DNA sequencing is necessary when a new mutation is present, when potentially indistinguishable digestion fragment patterns are present, such as the patterns from c.3412C>T and c.3413G>A, or when final identification of the sequence of a common mutation is required.

Recently, a multiphase strategy was described to screen for PXE mutations in the total coding sequence of the *ABCC6* gene.²⁵ PCR-RFLP and long-range PCR were used initially to detect the most common PXE mutations, c.3421C>T (p.R1141X) and ex23_ex29del, respectively. PCR and PCR-RFLP performed with five different restriction enzymes were used in a second phase to detect seven additional core PXE mutations. At this stage after a week's work, 41 to 78% of known mutant alleles in the DNA of a single patient could be screened,²⁵ depending on the genetic background of the patient.^{14,21,25,29} In the final stages, the entire coding sequences of patient DNAs with one or no PXE mutations were screened for mutations by denaturing high-performance liquid chromatography and Southern blotting. The test we have developed has several advantages over this approach. After only a single day's work, 62 to 74% of known mutant alleles^{14,21,25,29} in the DNA of a single patient can be screened. Because the PCR and enzymatic reactions are fewer in number, the test described here is much quicker, simpler, and easier to standardize and qualify to meet diagnostic regulatory requirements. In those patients in which mutations are not identified with the test, mutation scanning of other exons by nuclease analysis, by denaturing high-performance liquid chromatography, or by direct DNA sequencing can be performed in a more specialized laboratory.

Accurate and reliable genetic testing for PXE is needed to improve patient care and to facilitate further PXE research. Clinical laboratories offering genetic testing are subject to quality standards under Clinical Laboratory Improvement Amendments, but the analytical and clinical validity of the individual tests offered by the laboratory are usually determined by the individual laboratory and not subjected to external validation. As a leader in the development of effective diagnostics and treatment, PXE Interna-

tional seeks to have high quality, validated tests available to patients and providers. Thus, the mutation detection strategy described herein for PXE has been submitted to the Food and Drug Administration for approval as a medical device, currently the most stringent regulation for genetic tests. It is anticipated that a test kit with uniform performance characteristics will be available to providers and patients, decreasing interlaboratory variability and introducing total transparency about test specifications to clinicians, researchers, and patients. Use of the test in a Food and Drug Administration-approved form and in a CLIA-approved laboratory, rather than as a home-brew test, will provide assurance of genetic testing based on the use of standard protocols and quality-controlled reagents and will facilitate training of personnel in the use of standardized protocols.^{40,41} The use of such a test would also facilitate a unique approach to genetic testing for PXE. Standardized testing would allow PXE International, the test's sponsor, to integrate pretest genetic counseling, comprehensive informed decision-making, the opportunity to volunteer to have clinical results linked to a single phenotype database, posttest genetic counseling, and long-term follow-up, support, and education. Analysis of standardized test results compiled in a single database would facilitate refinement of the testing procedures and further discovery of mutations with the intent to develop allele-specific therapies.

Note

Subsequent to acceptance of this article for publication, a decision was made not to submit the PXE test described for Food and Drug Administration approval.

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