Types and Frequencies of Mutations in *ABCC6* that Characterize the Molecular Genetics of Pseudoxanthoma Elasticum (PXE)

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Introduction

Pseudoxanthoma elasticum (PXE) (OMIM 264800)

- is a systemic heritable disorder that affects the elastic tissue by progressive calcification and subsequent destruction of elastic fibers in skin, retina, and the cardiovascular system. While the cutaneous lesions have mainly cosmetic implications and major diagnostic relevance, it is the ocular and cardiovascular manifestations that can cause serious morbidity.
- The elastic tissue of skin in PXE was shown to contain an increased amount of gylcosaminoglycans.
- Mutations in the ATP-binding transmembrane transporter gene *ABCC6* were recently shown to cause PXE.
- The function of *ABCC6* and how perturbation of this function leads to PXE are still unknown.
- We performed a mutation screen in *ABCC6* through direct sequencing of 170 PXE chromosomes in 81 families with PXE, to gain insight into the molecular genetics of this clinically highly variable disease .
- In order to discriminate between different PXE associated chromosomes within the PXE families and to facilitate the detection of unique mutations we established PXE-associated haplotypes with genetic markers immediately flanking the *ABCC6* gene.

Methods

Collection of PXE-families and selection of samples for mutation screening

- 81 families with PXE represented 170 distinct disease chromosomes that were analyzed.
- At least one affected individual per family and a total of 4 unaffected members (that carried the reciprocal familial haplotype of their affected siblings) of different families were selected for direct sequencing.

Sequence and genotype analysis

- Fluorescent dideoxyterminator sequencing was carried out on ABI 377, ABI 3100 and ABI 3700 automated sequencing devices, on appropriate PCR-amplified exonic fragments of *ABCC6* using primers complementary to neighboring intronic sequence.
- Quality-score-based sequence comparisons were done using the "Sequencher" software tool version 4.05 (Gene Codes Corporation, Ann Arbor, MI).
- Using the *ABCC6* flanking microsatellite markers *D16B9621* and *D16B9622*, downstream and *D16S79* and *D16S764* upstream from the gene sequence genotyping and haplotyping was performed as has previously been published.

Sequence variants were considered to be phenotype modifying when

- *ABCC6* sequence variants result in nonsense or splice site alterations that
  - cosegregate with the disease haplotype in affected but not in unaffected members of all PXE families carrying the specific mutation, and
  - that are not detected in 200 control alleles of unrelated and unaffected individuals.
- Nucleotide variations result in amino acid substitutions that
  - firstly cosegregate with the disease phenotype and haplotype in all PXE pedigrees carrying it
  - secondly no other potentially disease-causing and familial haplotype specific allelic variant is identified by complete sequence analysis of *ABCC6*,
  - thirdly the variant was not found in the panel of 200 control chromosomes and
  - fourthly the amino acid substitution involved a conserved amino acid.

Results

Haploytping

- For mutation screening it was assumed that each distinct PXE haplotype was indicative of a different "founder" mutation. In this way representatives of each PXE haplotype were screened for mutation by direct sequencing.

Mutation screen

- The mutation detection rate was 97% (165/170 chromosomes) by direct sequencing
- Identification of a total of 59 distinct mutations of which 43 are novel variants. Of the 59 mutations
  - 32 are missense mutations, 8 are nonsense mutations, 6 are likely splice site point mutations, 1 is a small insertion, 7 are small deletions, 5 are large deletions.
- While most mutations are single variants the five most frequent PXE mutations (*R1141X*, *R1164X*, *Q378X*, c.2787+1G>T and e23-29del) account for almost 50% of PXE mutations in our family cohort.
- Most chromosomes that carry these five frequent PXE mutations have related haplotypes for each mutation, suggesting that these mutations originate from single founder mutations.
- Interestingly, the vast majority of mutations lies within intracellular domains of ABCC6. We found 4 missense mutations to be located in transmembrane domains.
- Nucleotide variations that cause amino acid substitutions located in extracellular domains do not cosegregate with any PXE haplotype and therefore not with the PXE phenotype.
- We demonstrated that gene conversion from one of the *ABCC6* pseudogenes is the source for the *Q378X* mutation in the *ABCC6* gene.
- By excluding known pseudogenes from amplification within the ABCC6 duplication region through allele specific primer pairs, we did unmask a G to T nucleotide substitution at position c.373 that results in premature termination of the protein at amino acid position 125 (*E125X*).
- The intronic and exonic polymorphisms c.345+12T>C, c.474+13G>A, c.794+36A>C, *K281E, I319V* were not found in recently characterized *ABCC6*-pseudogenes and provide evidence for additional uncharacterized pseudogenes within the duplications of the *ABCC6* region.

Table 1: Type and frequency of mutations in *ABCC6* identified in 170 disease chromosomes of 81 PXE families
Legend: RFLP: restriction fragment length polymorphism; Present or P: this study; E: exon; I: intron; cz: compound heterozygous; hz: homozygous. References: A: (Cai et al. 2001)), B: (Meloni et al. 2001)), C: (Le Saux et al. 2001)), D: (Ringpfeil et al. 2000)), E: (Le Saux et al. 2000)), F: (Bergen et al. 2000)), G: (Struk et al. 2000)), H: (Pulkkinen et al. 2001)), I: (Chassaing et al. 2004)), J: (Hu et al. 2003b)), K: (Ringpfeil et al. 2001))

Type of mutation	Nucleotide variant	Amino acid change	Exon Intron	Reference	RFLP: *Restriction site change*	RFLP: *PXE-allele size (bp)*	RFLP: *Wild type allele size (bp)*	Mutation frequency: %	Mutation frequency: (n/170)	Families (n/81)	Allelic state
Nonsense mutations (n = 8)	c.373G>T	*E125X*	E04	Present	*Bfa* I gain	235, 427	662		1	1	cz
	c.1132C>T	*Q378X*	E09	P, (A)	*Pst* I loss	365	230	3,5	6	5	cz, hz
	c.1552C>T	*R518X*	E12	(B), P	*Fok* I loss	387	215		1	1	cz
	c.2162G>A	*W721X*	E17	Present	*BstN* I loss	234	174		1	1	cz
	c.2304C>A	*Y768X*	E18	(C), P	*Bst217* I loss	312	192		1	1	cz
	c.2524C>T	*Q842X*	E19	Present	*Pst* I loss	349	175		1	1	cz
	c.3421C>T	*R1141X*	E24	P, (D, E, F)	*Bsl* I loss	123	111	26	45	38	hz, cz
	c.3490C>T	*R1164X*	E24	P, (G)	*BstU* I loss	95	64	5,3	9	8	hz, cz
Missense mutations (n = 32)	c.386G>A	*G129E*	E04	Present	*Mly* I loss	388	248		1	1	cz
	c.743C>T	*L248F*	E07	Present	*Tsp509* I gain	141	259		1	1	cz
	c.951C>A	*S317R*	E08	Present	*Hinf* I gain	217	268		1	1	cz
	c.1064T>G	*L355R*	E09	Present	*Fok* I gain	161	295		1	1	cz
	c.1091C>G	*T364R*	E09	(H), P	no restr. site				1	1	cz
	c.1108A>G	*N370D*	E09	Present	no restr. site				2	2	cz
	c.1171A>G	*R391G*	E09	(I), P	*BsrG* I loss	148	94		2	2	cz
	c.1192A>G	*S398G*	E10	Present	*Msp* I gain	217	273		1	1	cz
	c.1484T>A	*L495H*	E12	Present	*Bsu36* I loss	387	233		1	1	cz
	c.1553G>A	*R518Q*	E12	(C), P	*Sty* I gain	164	206	2,4	4	3	hz, cz
	c.1652T>C	*F551S*	E13	Present	no restr. site				1	1	cz
	c.1781C>T	*A594V*	E14	Present	*Ava* II gain	176	262		2	2	cz
	c.2030T>C	*L677P*	E16	Present	*Sty* I loss	226	110		1	1	cz
	c.2278C>T	*R760W*	E18	Present	*TspR* I gain	219	269		1	1	cz
	c.2294G>A	*R765Q*	E18	(J), P	*Sma* I loss	312	200		1	1	cz
	c.2419C>T	*R807W*	E19	Present	*Nla* III gain	252	286		1	1	cz
	c.2420G>A	*R807Q*	E19	Present	*Bsl* I loss	104	67		1	1	cz
	c.3168C>A	*D1056E*	E23	Present	*HpyCH4* IV loss	533	268		1	1	cz
	c.3340C>T	*R1114C*	E24	Present	*Hga* I loss	322	215		1	1	cz
	c.3362C>T	*S1121L*	E24	Present	no restr. site			1,7	3	2	hz, cz
	c.3412C>T	*R1138W*	E24	(D), P	*Msp* I loss	323	183		1	1	cz
	c.3413G>A	*R1138Q*	E24	(D, E), P	*Msp* I loss	323	183	1,7	3	3	cz
	c.3491G>A	*R1164Q*	E24	Present	*BstU* I loss	95	64		1	1	cz
	c.3661C>T	*R1221C*	E26	Present	no restr. site				2	2	cz
	c.3703C>T	*R1235W*	E26	Present	*BsrB* I loss	258	131		2	1	hz
	c.3904G>A	*G1302R*	E28	(C, J), P	*Msp* I loss	214	187		1	1	cz
	c.3907G>C	*A1303P*	E28	(C, J), P	no restr. site				1	1	cz
	c.3941G>A	*R1314Q*	E28	(C), P	*Pst* I gain	175	310		1	1	cz
	c.4015C>T	*R1339C*	E28	P, (G)	*BsiHKA* I gain	234	371	6,5	11	7	hz, cz
	c.4016G>T	*R1339L*	E28	Present	*Hha* I loss	310	212		1	1	cz
	c.4016G>A	*R1339Q*	E28	Present	*Hha* I loss	310	212	3	5	4	hz, cz
	c.4069C>T	*R1357W*	E29	Present	*Aci* I loss	233	200		1	1	cz
point mutations alternative splicing (n = 6)	c.2787+1G>T	Loss of ss	I21	(E), P	*BstE* II loss	305	213	3	5	5	cz
	c.3507-1G>A	Loss of ss	I24	Present	*Bsl* I loss	217	151		2	1	hz
	c.3735G>T	*E1245D*, loss of ss	E26	Present	*Hph* I loss	198	102		1	1	cz
	c.3735G>A	*E1245E*, loss of ss	E26	Present	*Hph* I loss	198	102		1	1	cz
	c.3736-1G>A	Loss of ss	I26	(D), P	*EcoN* I loss	297	223		1	1	cz
	c.3883-6G>A	(Gain of ss *A1295fsX1311*)	I27	Present	*BstX* I gain	98, 273	371		1	1	cz
Small deletion: in-frame del (n = 3)	c.179-187del	*R60_Y62del*	E02	(H), P	*Nci* I loss	302	96, 215		1	1	cz
	c.3106del-TTT	*F1036del*	E23	Present	*Taq* I gain	205, 325	530		1	1	cz
	c.3141del-CTT	*F1048del*	E23	Present	*Bsl* I gain	90, 108	201		1	1	cz
Small deletion / insertion: frame shift (n = 4)	c.105delA	*S37fsX80*	E02	Present	*MspA1* I gain	171	311		1	1	cz
	c.3769-3770insC	*L1259fsX1277*	E27	Present	*Mwo* I loss	184	114, 69		2	2	cz
	c.3775delT	*G1259fsX1277*	E27	(E, F), P	*Msp* I gain	27, 118	145, 151		2	2	cz
	c.3912delG	*S1305fsX1358*	E28	Present	*Bsl* I loss	118	29, 90		1	1	cz
Small deletion: altern. splicing	c.3307-3_-38del-3_-5insAGA	Loss of ss	I23	Present	Fok I loss	278	247		1	1	cz
Large deletion (n = 5)	c.2996_4208del	I1000_S1403del W1404fsX1463	E23-29	P, (C, J, K)				11	19	19	cz
	c.1-2787del	M1_R929del	E01-21	Present					1	1	cz
	delABCC6 ABCC1	M1_V1503del	E01-31	Present					1	1	cz
	delABCC6 ABCC1 MYH11, +5 genes	M1_V1503del	E01-31	Present					1	1	cz
	delABCC6 ABCC1 MYH11, +7 genes	M1_V1503del	E01-31	Present					1	1	cz

Table 2: Chromosomal founder haplotypes associated with frequent (≥3%) PXE mutation alleles.

Mutation	PXE haplotype (allele size in base pairs): *D16B9621*	*D16B9622*	*D16S79*	*D16S764*	Number of PXE chromosomes	Origin of families
c.3421C>T *R1141X* (n= 45)	323	139	163	133	17	USA (14), D (1), CH (2)
	323	139	163	137	6	USA (5), South Africa (1)
	323	139	153	133	7	USA (6) CH (1)
	323	139	153	137	3	USA (3)
	323	139	161	133	3	USA (2), D (1)
	323	139	153	129	2	USA (1), GB (1)
	323	139	165	141	2	USA (2)
	323	139	165	137	1	USA
	323	139	163	141	1	USA
	331	139	167	137	1	USA
	323	163	163	133	2	CH
g30906_47323del c.2996_4208del *I1000_S1403del W1404fsX1463 E23-29del* (n= 19)	323	165	163	133	11	USA (families of British/Irish descent)
	323	165	163	137	3	USA
	323	165	153	137	2	USA
	323	165	153	129	1	USA
	323	165	159	133	1	USA
	323	165	null	141	1	CH
c.3490C>T *R1164X* (n= 9)	333	165	163	129	2	USA (British desc.), GB
	331	165	163	129	1	USA (British desc.)
	331	165	163	133	1	USA (British desc.)
	323	165	null	137	1	USA
	315	165	null	137	1	USA
	323	171	153	133	1	USA
	323	165	157	133	1	USA (asian desc. same fam. as below)
	323	161	161	137	1	USA (asian desc. second allele)
c.4015C>T *R1339C* (n= 11)	333	169	153	129	8	South Africa (Dutch desc.)
	333	169	161	133	2	Mexico (Dutch desc.)
	347	139	153	133	1	Italy
c.4016G>A *R1339Q* (n = 5)	331	165	153	133	2	USA
	323	139	155	133	3	CH
c.2787+1G>T *Splice donor site* (n= 5)	333	157	153	137	2	USA
	331	157	153	137	2	USA
	331	157	null	137	1	USA
c.1132C>T *Q378X* (n= 6)	333	165	null	137	2	USA (Italian desc.)
	323	165	null	137	1	USA (Italian desc.)
	323	163	161	137	1	USA (Asian desc.)
	323	165	153	137	1	USA (Irish desc.)
	323	165	163	137	1	D
c.1553G>A *R518Q* (n = 4)	323	173	157	137	2	CH (homozygous alleles)
	323	173	159	137	2	USA, CH

Conclusions

- Mutation screening by direct sequencing provides the best mutation detection rate (here 97%) in comparison with other methods.
- Analysis of the topology of PXE mutations in the ABCC6 protein clearly shows a distinct distribution of mutations to particular protein domains.
- The most frequent PXE mutations originate from common founder alleles.
- The types of mutations observed (in-frame termination codons, small and large deletions and their homozygous and compound heterozygous cosegregation with the disease phenotype) suggest a loss-of-function as the molecular mechanism for PXE compatible with the recessive mode of inheritance of the disease.
- There is no clear relationship between genotype and phenotype in PXE, that would allow prediction of the clinical outcome based on the type of *ABCC6* mutations identified.
- While different mutations have the potential to affect ABCC6 function to varying extents, any such variation does not appear to be a rate limiting factor in phenotypic outcome.