Clinical and genetic aspects of pseudoxanthoma elasticum
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Chapter 9

General discussion
To obtain more insight in the inheritance, the clinical expression, and the molecular pathology of PXE, we performed clinical and molecular studies in patients with PXE and their family members. In this chapter our main findings will be discussed, followed by recommendations for future research.

INHERITANCE

Most PXE cases are sporadic, but in the past both autosomal recessive (AR) and, less frequently, autosomal dominant (AD) pedigrees were reported [1]. To investigate whether AD inheritance really exists, we scrutinized the literature on the subject and studied possible AD pedigrees in our own patient population. In the literature we found only three families with a convincing diagnosis of PXE in two successive generations [2-4] and none with PXE in three or more generations.

Our own patient population contained three families with putative AD inheritance. Two of them consisted of a PXE patient, in whom we only found one mutation, and a parent with respectively mild ophthalmic and mild dermatologic PXE signs. In the third family a definite diagnosis of PXE could be made in a mother and three of her eight children (Figure 4 in chapter 2). Using denaturing high-performance liquid chromatography (dHPLC) screening, only one ABCC6 mutation (c.Arg1459Cys) was found and linkage studies in combination with clinical examination of all of the eight sibs was not in accordance with AR inheritance. In conclusion, the latter family was the only one in which AD inheritance seemed most likely. However, we recently sequenced the entire gene and we found a second mutation in the three severely affected sibs, contradicting AD inheritance (unpublished results). In addition, we examined family members in the third generation of this family, who carried the c.Arg1459Cys mutation, but did not have clinical signs of PXE. In this family several questions remain unresolved. We did not find a second mutation in the affected mother. If the mother had a second mutation, the linkage study shows that two clinically normal sibs should also have two mutations. Did they have reduced penetrance? On the other hand, two sibs, who only inherited one c.Arg1459Cys mutation (and a normal allele from father), had angioid streaks. Is the heterozygous mutation in these sibs and in the mother sufficient for the clinical expression? Is another gene involved in these persons? Recently, in the literature two sibs with PXE-like skin abnormalities were reported to have one ABCC6 mutation and one GGCX mutation [5]. We analysed GGCX in our family, but we did not find any mutation. In conclusion, also in this family AD inheritance became less likely, but the exact inheritance pattern remains unclear. In all other families inheritance could have been AR with mild features in heterozygotes or with pseudodominance. Since our report, no other families with AD inheritance have been described and other authors confirmed that inheritance is probably always AR [6-8].
PHENOTYPIC VARIATION

It is well known that the phenotype of PXE can be very variable, but not much was known about the clinical variation within one genotype. It is hard to find a large group of patients with the same genotype, because the prevalence of PXE is low, the inheritance is AR and it can be caused by over 200 different mutations. We had the opportunity to examine 15 patients from a genetically isolated population. These patients were all homozygous for the same c.3775delT mutation in *ABCC6*. Skin signs varied from severe abnormalities around the age of 30 years to no abnormalities around age 60. The severity of the skin signs did not show a clear relation with age, in contrast with the eye abnormalities. Visual acuity was at least 0.7 in the worst eye of the five patients under the age of 50 years and at most 0.5 in the best eye of all six patients older than 56 years. Five out of 15 patients had cardiovascular problems. There was no marked correlation between severity of skin, eye or cardiovascular abnormalities. The phenotypic variation within this population can not be explained by different genotypes at the *ABCC6* gene, so other genes and/or environmental factors must play a role.

In the literature several genetic factors, potentially modifying the PXE phenotype, were reported: variations in the gene for xylosyltransferase II (XT-II) [9], promoter polymorphisms of the *SPP1* gene [10]; polymorphisms in the genes *CAT*, *SOD2* and *GPX1*, encoding for antioxidant enzymes [11]; serum concentrations of the calcification inhibitor matrix Gla protein (MGP) and a certain MGP haplotype [12]. As environmental factor high calcium [13] and/or high magnesium intake [14] could perhaps influence disease severity. Three of six patients, who were treated with the phosphate binder aluminum hydroxide, showed improvement of skin lesions [15]. The results of all of these studies were not yet confirmed by others. We did not test these factors yet in our patients with the c.3775delT mutation.

From the same genetically isolated population we examined 44 heterozygous carriers of the c.3775delT mutation. In the literature several signs and symptoms of PXE, especially abnormalities in skin biopsies, had been reported in persons, who were (probably) heterozygous for a single *ABCC6* mutation. We did not find any PXE skin or eye sign in our heterozygotes, even not in 68 skin biopsies when compared to control biopsies. Why did we find no PXE signs in heterozygotes, while others did? There are several possibilities:

1. Expression in heterozygotes might be different for different genotypes.
2. Putative heterozygotes could still have a so far undiscovered second *ABCC6* mutation.
3. For reliable results, observers should be masked for the genotype and control persons should be included. This was not always the case in other studies.
4. Signs in heterozygous persons could be rare. The reported cases in the literature could be a small selection and not representative for the whole group.

Others also found that the risk of cardiovascular disease was increased in heterozygotes [16-18]. In our study the prevalence of a positive cardiovascular disease history was equal between homozygous (33%) and heterozygous (32%) family members, but the small number and absence of a control population make it impossible to come to more definite conclusions.
CLASSIFICATION

Making a correct diagnosis of PXE in a patient is important for several reasons. Preventive measures can be taken to lower the risk of complications, such as retinal hemorrhage, gastrointestinal hemorrhage and cardiovascular disease [19, 20]. A reliable diagnosis is also important for clinical trials, for comparing research results, for genetic counseling and if a therapy for PXE will become available in the future.

The most recent PXE classification dated from 1994 and was the result of a consensus conference [21]. Based on several major and minor clinical criteria, patients could be placed in category I (definite diagnosis) and category II (uncertain diagnosis). Since then, the gene for PXE was found, so that mutational analysis can now be included in the revised diagnostic criteria. Because this analysis is not available for everyone, mutations are still not found in all PXE patients, and the presence of mutations is not sufficient for making the diagnosis of PXE, clinical criteria remain important. In chapter 4, we propose an updated PXE classification system. The most important modifications with regard to the 1994 classification system are discussed here. In our experience, one of the main problems in diagnosing PXE in part of the patients is to decide whether there are skin abnormalities which point to PXE. The skin abnormalities are variable, can be mild and sometimes resemble other skin diseases. Therefore, we added pictures of some variations in skin abnormalities in PXE (figure 1a-d in chapter 4) and of solar elastosis (figure 3a,b), which can resemble the PXE skin signs. In our diagnostic criteria we included ophthalmologic signs (comets and pigmented wings), which seem to be specific for PXE, as major criteria. We propose guidelines for patient examination and exclusion of the most important differential diagnoses by additional investigations, in case mutational analysis of ABCC6 is negative or not available. Based on our criteria, patients can be classified as having definite, probable, possible or no PXE. A definite diagnosis can now be made in part of the patients, who did not have definite PXE according to the previous classification system. In our classification, persons with all possible combinations of signs and symptoms can be placed into a category, in contrast with the 1994 classification.

A limitation of our classification system still is that the sensitivities and specificities of the different clinical PXE signs are largely unknown. This might be solved when a large group of 200 or more homozygous PXE cases can be carefully examined according to a strict protocol by experienced investigators. Because there is up till now no golden standard for the diagnosis of PXE, it remains possible that different PXE experts disagree about the relevance of the different criteria and about the definition of definite, probable and possible PXE.

MOLECULAR GENETICS

In 2000, we (chapter 5) and others [22, 23] found that PXE is caused by mutations in the ABCC6 gene on chromosome 16p13.1. The gene comprises 31 exons and spans about 73 kb of genomic DNA. The ABCC6 protein consists of 1503 amino acids and contains 17 transmembrane spanning domains and two intracellular nucleotide binding folds (NBFs). In
In total, we identified 40 different mutations, of which 19 were novel, in 203 alleles. All types of mutations (missense, nonsense, splicing alterations, insertions and deletions) were present. The large majority of mutated alleles had mutations, which can be predicted to result in absent or severely dysfunctional protein. Only 8.4% of alleles had a missense mutation. The mutations were not evenly distributed over the gene. The three mutation hot spots were both NBFs and the eighth cytoplasmic loop, suggesting that these are functionally important domains. Three mutations were found relatively frequently, c.3421C>T (p.Arg1141X) in 33% of mutated alleles, c.3775delT in 14%, and a deletion of exons 23-29 in 13%. In 87% of probands with a clinical diagnosis of PXE at least one mutation was found. Our missing rate per allele was 35%. Possible explanations for this relatively high missing rate are:

1. Part of the patients might not have PXE. We did not have detailed clinical information of all patients.
2. After screening the gene for common mutations, all exons were screened by dHPLC. We could have missed some missense mutations, mutations in the promoter region and in introns and heterozygous deletions. One or more missed mutations might have a relatively high frequency in our population.
3. Digenic inheritance could play a role in some patients, in whom we found one mutation, as suggested by Li et al. [5].

Neither we, nor several other authors, could find a genotype-phenotype correlation [6, 24-26], although Gheduzzi et al. suggested that nonsense mutations were more frequently associated with generalized involvement [24]. Also a significantly younger age at diagnosis and a higher number of affected organs in the case of mutations that lead to an absence of (functional) ABCC6 protein were described [27].

In chapter 8, we studied the most frequent mutation, c.3421C>T (p.Arg1141X), in more detail in 16 patients, who were homozygous, compound heterozygous or heterozygous for this mutation. The majority (17/19) of alleles with the mutation shared the same haplotype, which was not present in other patients or control persons, suggesting a common founder for the p.Arg1141X mutation in our population. Patients, who were homozygous for the mutation, did not have detectable ABCC6 mRNA or ABCC6 protein in cultured dermal fibroblasts. This might be due to nonsense-mediated mRNA decay and suggests that PXE in these patients is caused by complete loss of ABCC6 function. We did not find indications for a specific phenotype correlated to this mutation, but the number of patients was small and only three were homozygous for the mutation.

**FUTURE RESEARCH**

**Further analysis of the phenotype**

The phenotype of PXE can be very variable. In chapter 4, we propose criteria to be able to make a diagnosis of definite, probable, possible or no PXE, but it remains to be elucidated what the
full spectrum of clinical variation is in persons with two \textit{ABCC6} mutations. Related to this issue, the exact prevalence of PXE is still unknown. Trip \textit{et al.} (2002) found the frequent Arg1141X in 0.8% of 1057 control subjects \cite{16}. We found this mutation in 23% of disease alleles (chapter 7). This means that PXE carrier frequency in the normal population could be as high as about 1 in 30. Consequently, about 1 in 3600 persons could be homozygous. Why are the reported prevalences much lower? The fact that the mean age of onset of PXE seems to be around 13 years can not fully explain this. Are many patients clinically unrecognized? Is there non-penetrance in part of the patients? It would be interesting to perform \textit{ABCC6} analysis in for example 10,000 participants in a population based study to establish the total \textit{ABCC6} mutation carrier frequency. It would also be interesting to establish the frequencies of the different skin, eye and cardiovascular signs in persons, who where only selected on the presence of two \textit{ABCC6} mutations (and not on clinical criteria), to prevent selection bias as much as possible. Especially the risk of cardiovascular disease is now largely unknown, but very relevant for patients. Such a study might demonstrate non-penetrance. The data could also lead to improvement of the classification system.

A similar clinical study can be done in a large group of heterozygous persons with different \textit{ABCC6} mutations to answer the question why some authors found clinical abnormalities in heterozygotes and others did not. More data are also needed to establish the risk for cardiovascular disease in this group.

Yet another question is what causes the phenotypic variation? Up to date, no clear genotype-phenotype correlation could be found and the phenotype is also very variable within one genotype. Consequently, other genetic and/or environmental factors must play a role. As discussed in chapter 1.3, several factors have been suggested, but more research is necessary to confirm these findings and to find other factors. The recent finding of Larusso \textit{et al.} (2009) that a diet high in magnesium prevented connective tissue mineralization in \textit{Abcc6} knock-out mice \cite{14}, warrants further research into the role of magnesium in humans. The more we know about the pathophysiology of PXE, the more new potential modifier factors may emerge. As long as there is no causal therapy for PXE, knowledge about modifier factors (like diet) could have important therapeutic implications.

\textbf{Molecular genetic research}

Mutation detection needs to be continuously further improved, as mutations can not yet be found in all patient alleles. These could be mutations in the promoter region, in introns, heterozygous deletions, or mutations outside the gene, which influence gene expression. Up to date, the involvement of a second PXE gene seems unlikely, but this can not be completely excluded yet. In addition, the possibility of digenic inheritance (with the \textit{GGCX} gene or other \textit{GGCX} genes) could be further investigated.

\textbf{Research into the pathophysiology of PXE}

One of the main unresolved queries in PXE research is how \textit{ABCC6} mutations lead to the PXE phenotype. Recently Borst \textit{et al.} (2008) \cite{28} suggested that the \textit{ABCC6} protein transports a
vitamin K derivative into the blood to supply peripheral tissues. Vitamin K is needed there for the gamma-carboxylation of (among others) matrix gla-protein (MGP), which is important for the prevention of tissue calcification. PXE may be caused by a local shortage of vitamin K4 or K7 in the periphery. Vitamin K suppletion studies in Abcc6 knock-out mice to test this hypothesis are still pending. If the hypothesis is correct, suppletion with certain vitamin K subtypes could be an effective therapy for PXE, which subsequently could be tested by means of clinical trials. If the hypothesis is incorrect, further studies in the mouse model hopefully will shed light on the disease mechanism. These could include further research into the vitamin K and/or MGP metabolism. Analysis of the different metabolites in tissues of the Abcc6-/- mouse might point to the right direction. Another possibility is microarray analysis to compare gene expression in the relevant tissues between Abcc6-/- and wild type mice. Significant differences could give information on the disease mechanism.
REFERENCES


