Analysis of ABCC6:

Elucidation of the Molecular Pathology of

Pseudoxanthoma Elasticum

Xiaofeng Hu

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Analysis of ABCC6: Elucidation of the Molecular Pathology of Pseudoxanthoma Elasticum

ABCC6 Analyse:
Opheldering van de Moleculaire Pathologie
van Pseudoxanthoma Elasticum

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For my mother, and in the memory of my father

To Baoxian and Jiyao

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Pseudoxanthoma Elasticum: A clinical, histopathological and molecular update. Hu X, Plomp A, van Soest S, Wijnholds J, De Jong PTVM, Bergen AAB.
Surv Ophthalmol. 2003; 48: 424-438

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ABCC6/MRP6 mutations: Further insight into the molecular pathology of pseudoxanthoma elasticum. Hu X, Plomp A, Wijnholds J, ten Brink J, van Soest S, van den Born LI, Leys A, Peek R, De Jong PTVM, Bergen AAB. Eur J Hum Genet. 2003;11(3):215-224

Chapter 3
Analysis of the frequent R1141X mutation in the ABCC6 gene in pseudoxanthoma elasticum. Hu X, Peek R, Plomp A, ten Brink J, Scheffer G, van Soest S, leys A, de Jong PTVM, Bergen AAB. Invest Opthalmol Vis Sci. 2003;44 (5):1824-1829

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Efficient molecular diagnostic strategy for ABCC6 in pseudoxanthoma elasticum. Hu X, Plomp A, Grogels T, ten Brink J, W Loves, Mannens M, de Jong PTVM, Bergen AAB. (submitted)

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Frequent mutation in the ABCC6 gene (R1141X) is associated with a strong increase in the prevalence of coronary artery disease. Trip MD, Smulders YM, Wegman JJ, Hu X, Boer JM, ten Brink J, Zwinderman AH, Kastelein JJ, Feskens EJ, Bergen AAB. Circulation. 2002;106(7):773-775

Chapter 6
Does autosomal dominant PXE exist? Plomp A, Hu X, de Jong PTVM, Bergen AAB (Accepted for Am J Med Genet)

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Chapter 1

INTRODUCTION

Chapter 1 *Part 1*

Introduction

Pseudoxanthoma elasticum (PXE) is an inherited disorder of connective tissue, in which abnormally calcified and fragmented elastic fibers develop in skin, Bruch's membrane in the retina, and vessel walls.[1-3] The skin lesions include yellowish papules and plaques, mainly on the lateral side of the neck and on flexural areas of the body. Ocular signs are peau d'orange of the retina, followed by angioid streaks which are cracks in Bruch's membrane in the retina. Angioid streaks are associated with subretinal neovascularization and hemorrhage, eventually leading to disciform scarring and severe visual loss.

Mutations in the *ABCC6* (Formerly called multidrug resistance protein 6, MRP6) gene have been found in subjects with sporadic PXE, autosomal recessive (AR) PXE, and potentially autosomal dominant (AD) PXE.[4-6] *ABCC6,* a member of the ATP-binding cassette (ABC) gene subfamily C, is 1503 amino acids long and has a mass of 165 kD.[7,8] The ABCC6 protein contains three hydrophobic membrane spanning domains, 17 transmembrane spanning helices and two evolutionarily conserved ATP- binding folds.[8] ABCC6 is highly expressed in kidney and liver in humans. Low ABCC6 expression was found in the normal human tissues most directly affected by PXE.[4,8] Human ABCC6 transports glutathione conjugates, including leukotriene-C4 and N-ethylmaleimide S-glutathione.[10] For three missense mutations, loss of transport activity appears to be directly responsible for the development of the PXE phenotype.[10] Recently, structural analysis of urinary sulfated polyanions in PXE patients showed that the metabolism of proteoglycans is altered in PXE.[11]

Recent progress in the research on structure and function of the *ABCC6* gene has opened up new avenues towards understanding the molecular pathology of PXE. However, many questions remain to be clarified. The functional consequences of mutations in the *ABCC6* gene have yet to be fully elucidated. The reason for the variable expression of the disease in patients and carriers of PXE is still not clear. The mode of inheritance of PXE is not completely understood. How aberrant transport by ABCC6 results in the accumulation of abnormal elastic fibers remains a puzzling question.

The aim of this thesis is to explore the molecular pathology of PXE, i.e. the interpretation of pathological findings based on molecular genetic findings. In the 1st chapter, the current knowledge of PXE is reviewed from a clinical, histological and molecular point of view. In the 2nd chapter, we describe the mutation spectrum of the *ABCC6* gene in 59 Dutch PXE patients and we report selected data on PXE family studies. Next, in chapter 3, the effect of a frequent mutation (R1141X) in the *ABCC6* gene in PXE patients is characterized in more detail by scrutinizing mRNA transcripts and protein in skin fibroblasts. In chapter 4, a practical and effective molecular diagnostic strategy, and its consequences for genetic counselling of PXE patients are described. In the 5th chapter, we describe that the most frequent mutation, R1141X, is a risk factor in patients with coronary artery disease. In chapter 6, possible existence of AD inheritance in PXE is discussed by evaluation of clinical features, pedigree data, haplotype and mutation analyses. Chapter 7 describes the production of monoclonal antibodies against the ABCC6 protein and the use of these antibodies for localization of ABCC6 protein in normal human tissues. The main findings and their implications for the molecular mechanisms of PXE, as well as prospects for further studies, including mouse models for PXE, are presented in the concluding chapter 8.

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Chapter 1 *Part 2*

Pseudoxantoma Elasticum: A clinical, histopathological and molecular update

Outline

CURRENT RESEARCH

EDWARD COTLIER AND ROBERT WEINREB, EDITORS

Pseudoxanthoma Elasticum: A Clinical, Histopathological, and Molecular Update

Xiaofeng Hu, MD,[1] Astrid S. Plomp, MD,[1,2] Simone van Soest, PhD,[1] Jan Wijnholds, PhD,[1] Paulus T.V.M. de Jong, MD, PhD, FEBO, FRCOphth,[1,3,4] and Arthur A.B. Bergen, PhD[1,2]

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Abstract. Pseudoxanthoma elasticum is an autosomally inherited disorder that is associated with the accumulation of mineralized and fragmented elastic fibers in the skin, Bruch's membrane in the retina, and vessel walls. The ophthalmic and dermatologic expression of pseudoxanthoma elasticum and its vascular complications are heterogeneous, with considerable variation in phenotype, progression, and mode of inheritance. Using linkage analysis and mutation detection techniques, mutations in the ABCC6 gene were recently implicated in the etiology of pseudoxanthoma elasticum. ABCC6 encodes the sixth member of the ATP-binding cassette transporter and multidrug resistance protein family (MRP6). In humans, this transmembrane protein is highly expressed in the liver and kidney. Lower expression was found in tissues affected by pseudoxanthoma elasticum, including skin, retina, and vessel walls. So far, the substrates transported by the ABCC6 protein and its physiological role in the etiology of pseudoxanthoma elasticum are not known. A functional transport study of rat MRP6 suggests that small peptides such as the endothelin receptor antagonist BQ123 are transported by MRP6. Similar molecules transported by ABCC6 in humans may be essential for extracellular matrix deposition or turnover of connective tissue at specific sites in the body. One of these sites is Bruch's membrane. This review is an update on etiology of pseudoxanthoma elasticum, including its clinical and genetic features, pathogenesis, and biomolecular basis. (**Surv Ophthalmol 48**:424–438, 2003.

Key words. ABCC6 ▫ ATP-binding cassette (ABC) transporter ▫ Bruch's membrane ▫ elastic fibers ▫ gene ▫ pseudoxanthoma elasticum ▫ PXE

Pseudoxanthoma elasticum (PXE) is an inherited disorder with multiple systemic manifestations, including abnormalities of the skin, Bruch's membrane (BrM) in the eye, and the vascular system.[49,60,73,91] The histopathological features of skin lesions are mineralization and fragmentation of elastic fibers.[104,143] Similar changes also occur in the elastic fibers of BrM in the retina of PXE patients eventually often resulting in angioid streaks, choroidal neovascularisation, and, consequently, loss of visual acuity.

PXE is so far incurable and appears to be present in all of the world's populations with an estimated prevalence of 1:70,000 to 1:100,000 live births.[134]

The prevalence of PXE may be higher than reported in literature due to the variable expression and penetrance as well as infrequent occurrence of the disease, which may result in insufficient awareness of medical specialists. Indeed, given the clinical heterogeneity and different modes of inheritance, it has been difficult to diagnose PXE accurately and to calculate correct genetic risks for genetic counseling purposes.

Recently, mutations in the ABCC6 gene have been implicated in PXE.[12,69,111] To date, ABCC6 mutations have been found in 80% of patients with PXE. Although the exact function of the PXE gene remains to be elucidated, these findings immediately enable more accurate diagnosis and genetic counseling. Abbreviations used throughout the manuscript are summarized in Table 1.

Clinical Features of PXE Patients

THE SKIN AND MUCOSAL MEMBRANES

Skin lesions are frequently seen in PXE patients and were initially described in 1881 by Rigal[110] and in 1896 by Darier.[27] In 1929 Groenblad and Strandberg recognized the combination of skin and eye abnormalities for the first time.[52,132]

The most common presentation of skin lesions involves ivory to yellowish-colored, raised papules varying in size from 1–3 mm. The papules may have a linear or reticular arrangement and may coalesce into plaques.[125] Sometimes larger confluent areas with deposits are seen, as well as areas with purpura and complete necrosis of the skin.[125] In many PXE cases, the skin becomes wrinkled and redundant, hanging in folds. These folds may become more marked during pregnancy.[59] Personally, we have also seen marked deep grooves in the skin at the corners of the mouth in the extension of the nasolabial folds and on the forehead in line with the nasal root in a number of PXE patients. Initially, the skin lesions erroneously were described as a form of cutis laxa.[59]

TABLE 1

Abbreviations and Definitions

ABC transporter	ATP-binding cassette transporter
ABCC	C sub-family of ABC transporters
ad or AD	autosomal dominant
ar or AR	autosomal recessive
AS	angioid streaks
ATP	adenosine-tri-phosphate
BrM	Bruch's membrane
BQ-123	a synthetic penta-peptide
EBP	elastin-binding protein
kb	kilobase
kD	kilodalton
MRP6	multidrug resistenace protein 6 (human)
Mrp6	multidrug resistance progein 6 (rat)
MYH11	myosin heavy chain gene
NBF	nucleotide binding fold
NH2	amino-terminal end of a protein
PXE	pseudoxanthoma elasticum
RPE	retinal pigment epithelium

On average, the diagnosis of PXE skin abnormalities is made at 22 years of age, after a mean delay of 9 years since first signs.[59] Our youngest case had skin lesions diagnosed at the age of 6 years. Skin abnormalities usually start on the lateral side of the neck (Fig. 1). Subsequently, they occur on the flexural areas such as armpits, antecubital and popliteal fossae, the inguinal region, and the periumbilical area. More rare is localization on the face. In one case, presenting with cutis laxa–like features, PXE osteomas were found in the skin.[19]

Clinically visible PXE-like skin lesions are not pathognomonic for PXE, because they also occur in late-onset focal dermal elastosis,[77] in beta-thalassemia,[6] in adult patients with deforming osteitis (Paget's disease) or osteoectasia,[115] in farmers exposed to saltpeter fertilizers,[20] in PXE-like papillary dermal elastolysis,[114] and in patients having had penicillamine therapy.[87] Isolated periumbilical skin lesions are called *periumbilical perforating PXE*, but there is no known relation with hereditary PXE.[121] The differential diagnosis also should include actinic dermal changes, disseminated lenticular dermatofibrosis with osteopoikilia, mediodermal and papillary elastolysis, and changes due to the l-tryptophane induced eosinophilia-myalgia syndrome.[59]

Apart from the skin lesions, similar yellowish abnormalities of the mucosal membranes have been reported on the inside of the lower lip, on the remaining oral mucosa including the sublingual one and on the soft palate, nose, larynx, stomach, bladder, penis, rectum, and vagina.[125]

Finally, the complete absence of skin or mucosal membrane lesions is no reason to exclude PXE.[75] We will discuss this below. This might, among reasons, be due to varying penetrance and expression of the disorder.

THE EYE

Ocular signs eventually develop in most patients with PXE. The usual sequence of developing eye abnormalities is peau d'orange or mottled hyperpigmentation of the retina, angioid streaks (AS), peripapillary atrophy with or without white glial tissue formation, and finally, subretinal neovascularization. The natural course of the latter often leads to a disciform scar in the macula that causes decreased visual acuity.

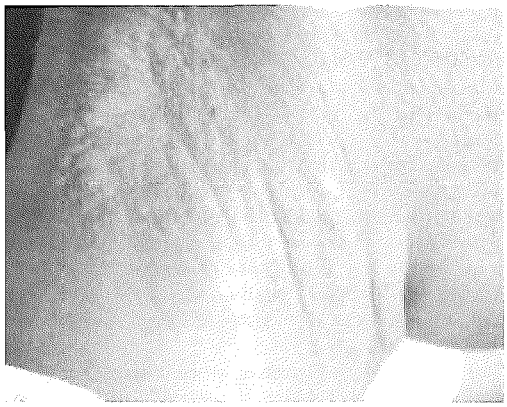

Fig. 1. Yellowish papules ("plucked chicken, goose pimples") and plaques on the right side of the neck of a pseudoxanthoma elasticum patient.

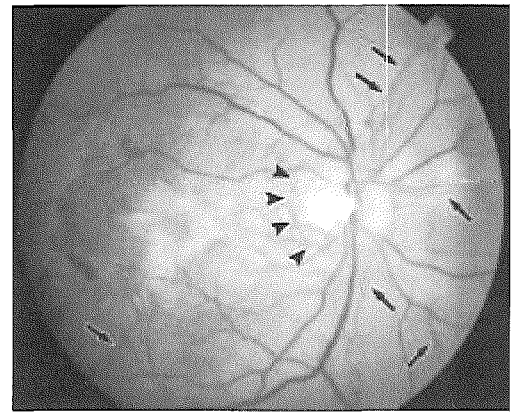

Fig. 2. Angioid streaks in the right fundus of a 47-year-old man with pseudoxanthoma elasticum. Arrows point to broad streaks, resembling larger vessels. On the left side of the disk arrowheads indicate more circumferential smaller streaks. There is a scar in the fovea leading to enhanced visibility of the luteal pigmentation. On its left are hemorrhages from a subretinal neovascular membrane.

Peau d' orange is more often observed than AS in young patients and this observation does suggest that peau d' orange may be a precursor to AS for many years.[130,139] Peau d' orange is caused by diffuse mottling of the RPE and deposition of yellow material, most prominent temporal to the fovea. However, the most common ocular sign reported in patients with PXE is AS (Fig. 2). Vice versa PXE was diagnosed in 86% of 58 patients with AS.[101] AS are broad, irregular, and red-brown to gray lines that on first glance resemble choroidal or retinal blood vessels. The streaks usually appear in the second to third decade of life. Fluorescein angiography can enhance the detection of early streaks.[21,131] In many patients there is a slow increase in length, width, and number of streaks. AS almost always develop bilaterally and they usually extend in a zigzag, radiating pattern towards the retinal periphery. Sometimes, connecting streaks concentric around the disc can be observed.

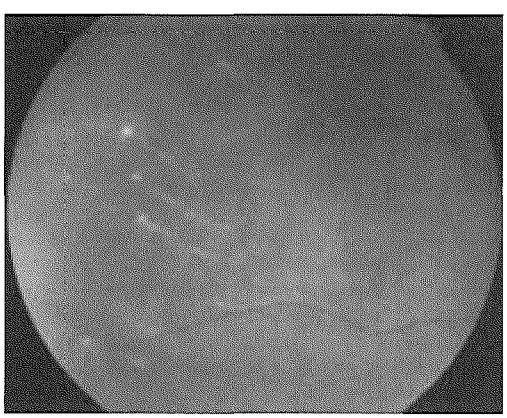

Fig. 3. Comet-like tails in the equatorial region of the right eye of a 47-year-old man with pseudoxanthoma elasticum. The head shows sometimes hyperpigmentation next to hypopigmentation and the tails are hypopigmented retinal pigment epithelium.

In the fundus, the peripapillary atrophy in PXE patients is sometimes seen as an atrophic helicoid shape, or as a white peripapillary ring. However, in our experience the atrophy may also have a cuboid form with radial extensions that may represent old AS. Atrophy of the RPE may eventually become so extensive around the disk and in the macular area that one can no longer see any signs of AS.[40] The latter may complicate ophthalmic screening for PXE. Among the ocular differential diagnosis of curvilinear hypopigmented or hyperpigmented AS can be mentioned in probably declining prevalence myopic lacquer cracks of the RPE, choroidal detachments and folds, choroidal ruptures after trauma, internal ophthalmomyiasis, as well as macroreticular retinal dystrophy. Angioid streaks may become less marked with time or disappear in conjunction with a generalized atrophy of the RPE and choroids[129] or reactive hyperplasia of the RPE as we have incidentally seen.

Angioid streaks are the only sign of PXE for many years in some patients.[121] Scholz found that 59% of 139 cases with AS had clinical evidence of PXE.[125] Connor reviewed 106 cases of AS and found that 80–87% of these had PXE.[23] Others reported that nearly 100% of patients would have AS after 20 years of

TABLE 2

Systemic Disorders in which the Presence of Angioid Streaks has been Mentioned

1.	abetalipoproteinemia
2.	AC hemoglobinopathy
3.	Acanthocytosis (abetalipoproteinemia, Bassen–Kornzweeg syndrome)
4.	Acromegaly
5.	Acquired hemolytic anemia
6.	Beta thalassemia minor
7.	Calcinosis
8.	Cardiovascular disease with hypertension
9.	Chronic congenital idiopathic hyperphosphatasemia
10.	Chronic familial hyperphosphatemia
11.	*congenital dyserythropoietic anemia type III (CDA-III)
12.	Cooley anemia
13.	diabetes
14.	Diffuse lipomatosis
15.	Dwarfism
16.	Epilepsy
17.	Facial angiomatosis (Sturge-Weber)
18.	Fibrodysplasia hyperelastica (Ehlers–Danlos syndrome)
19.	François dyscephalic syndrome (Hallermann–Streiff syndrome)
20.	Hemochromatosis
21.	hemolytic anemia (acquired)
22.	Hereditary spherocytosis
23.	Hypercalcinosis
24.	*hyperphosphatemia
25.	Lead poisoning
26.	*Marfan syndrome
27.	*Multiple hamartoma syndrome
28.	Myopia
29.	Neurofibromatosis
30.	Ocular melanocytosis
31.	Optic disc drusen
32.	Osteitis deformans (Paget disease)
33.	Pituitary tumor
34.	Previous choroidal detachment
35.	Pseudoxanthoma elasticum (Grönblad–Strandberg syndrome)
36.	Senile (actinic) elastosis of the skin
37.	Sickle cell disease (Herrick syndrome)
38.	Sturge–Weber syndrome
39.	thalassemia
40.	Trauma
41.	Thrombocytopenic purpura
42.	Tuberous sclerosis

*Overview of all disorders in which the presence of angioid streaks has been described. It is dubious if these all were real angioid streaks, but often that cannot be gathered from the articles. Those disorders in which angioid sreaks are most common or are most likely real angioid streaks have been marked with an asterisk.

Data collected from the following references. [3,4,44,118,120,146]

disease.[94] Although AS are most frequently found in PXE, they have also been described in many other ocular and systemic diseases (Table 2). In our view, there is only one fundus feature that seems to be typical for PXE: small, round punched-out lesions of the RPE in the mid-periphery of the retina with a diameter of about 125 □m leading to white dots, which may extend to the sclera, with a slightly depigmented tail in the RPE (Fig. 3). Gass coined the term *comet-like tails* for these.[43] This author described them as similar to the lesions seen in presumed ocular histoplasmosis (POHS) but in our experience the white dots in PXE seem to have a brighter white color with a pearly shine and they miss their tail in POHS. The lesions may also occur in the posterior pole of PXE eyes but there they often miss their comet-like tail and in that case resemble lesions in myopia and sickle cell disease.

Three different types of drusen have been described in PXE. Drusen of the optic disk seem to be most common and were found with ultrasonography in 21% of PXE eyes with AS and in 25% of eyes with AS in patients with no sign of PXE.[101] They thus might be more associated with AS than with PXE. Dense clustering of soft drusen in both macular areas of a woman at age 34, suspected of PXE, seems to be rare for age-related maculopathy. Perhaps even more rare is the published image of small drusen, estimated to be 125 □m in size, in a concentric circle at 250–500 □m around both optic disks of a 21-year-old man.[125]

Besides drusen, myopia was mentioned in 21% of autosomal dominant PXE cases from the literature.[102] However, without a well-selected control population one wonders if this is different from the 20% myopia over −0.5 diopter that we found in the population-based Rotterdam Study, be it that the latter was on subjects 55 years and over. Finally, the higher than normal prevalence of chorioretinal arteriovenous communications in four eyes of 27 cases with AS and PXE are another ocular sign of PXE.[126]

The expression or penetrance of the ocular manifestations of PXE may vary considerably within a single family.[127] Illustrative is one report[40] on six sisters, aged 44–66 years:

Case 1, eldest sister: Only "a small whitish fleck inferonasally to the macula in the left fundus," plus PXE skin lesions.

Case 2: Only angle-closure glaucoma without fundus signs.

Case 3: Pigment dispersion in the retina with atrophy and AS nasal to the disk and in the mid-periphery, plus PXE skin lesions.

Case 4: Disciform scars in both maculae, AS in the mid-periphery, PXE skin lesions.

Case 5: AS, fundus atrophy after a resolved macular hemorrhage, optic disk drusen and PXE skin lesions.

Case 6, the youngest sister: Full vision, extensive drusen mostly temporal to the macula without AS but positive PXE skin lesions.

Of their offspring, 2 out of 11 examined children had optic disk drusen but none showed PXE skin lesions.

We are not aware of deleterious effects of peau d'orange or comet-like tails on visual function. However, the visual prognosis for patients with AS is often poor. In 75% of patients with AS a disciform macular degeneration develops with formation of subretinal vessels and, eventually, discoid scar tissue resulting in considerable loss of central vision.[53,83,82,126] Indeed, decreased visual acuity occurs in a large number of individuals affected by PXE, but total blindness is rare.[28,37] In the general population, blunt and severe ocular trauma frequently results in choroidal ruptures with a subsequent disciform reaction. Subjects having PXE will develop choroidal ruptures already at lower impact forces, making protective eye wear even more advisable. For a more extensive overview of the ocular signs of AS we refer the reader to Clarkson and Altman.[21]

THE CARDIOVASCULAR AND LESS FREQUENTLY AFFECTED SYSTEMS

One gets the impression that the common feature of PXE in all other organ systems may be reduced to generalized calcification of tissues and vessels, resulting in abnormal brittleness and vascular occlusion. Cardiovascular complications in PXE are relatively frequent, but exact risks are not known. Cardiovascular symptoms and signs include angina pectoris, diminished pulse waves, hypertension, restrictive cardiomyopathy, mitral valve prolapse and stenosis, fibrous thickening of the endocardium and atrioventricular valves, and sudden death at younger age.[18,42,70,74,93,97,106] Apart from the latter, the most serious complication in PXE is accelerated arteriosclerosis. Arterioslerotic heart disease and hypertension at 4 years of age have been described.[122] Intermittent claudication, the most common cardiovascular symptom, was described as early as age 6, but usually does not occur until the third decade of life.[125]

In PXE patients, the compressibility of the carotid arterial wall was 44% higher than in control subjects. This compressibility was higher before age 40 years and declined after that in PXE patients contrary to a linear rise in control subjects. This was attributed to accumulation of proteoglycans in the vessel walls of PXE cases.[14]

In an overview of 200 PXE cases from the literature, the following percentages for prevalent abnormalities were given: disappeared or diminished peripheral vascular pulsations 25%, systemic hypertension 22.5%, angina pectoris 19%, intermittent claudication 18%, and gastrointestinal hemorrhages 13%.[38] In 40 beta-thalassemic patients, many arterial calcifications were found. In this group, 20% had PXE-like skin lesions and 52% AS so that the authors spoke of acquired PXE syndrome, also encompassing strokes.[1,2]

One quarter of PXE patients gets renovascular hypertension and echographic opacities due to calcification of arteries in kidneys, spleen, and pancreas, sometimes as early as 10 years of age.[24] These could be filed under "vascular complications," but also hepatic and splenic malformations have been documented in which the vascular genesis was unclear.[66] After retinal hemorrhages, the gastrointestinal ones are most frequent, leading in up to 15% of cases to hematemesis and melena.[41] These are due to calcification of elastic fibers in the thin-walled arteries located directly under the gastric mucosa.[75] Subarachnoid, nose, pulmonary, renal, bladder, and joint bleedings are less common[41,128] but in our experience a history of menometrorrhagias seems to be given more frequently. In the placenta of PXE women, more mineral precipitates and matrix-type fibrinoid was found on the maternal side[46] but most pregnancies and deliveries do not constitute a problem. The hemorrhages in PXE patients are generally said to result from calcified vessels and not from, for example, mucosal lesions. One rare case of bilateral 4-cm diameter necrotic breast tumors in a 64-year-old female PXE patient has been described.[148]

Histology and Pathology of PXE

THE SKIN

The classic histological picture of PXE skin is elastin abnormality in the mid-epidermis with normal morphology in the papillary and deep dermal layers.[87]

The elastin band undergoes swelling, granular degeneration, and fragmentation; splitting and curling of elastin fibers gives it the appearance of an iron wool scouring pad upon Von Kossa staining.[75,90,117] In the abnormal granular elastin matrix, calcium depositions ($CaCO_3$ and $CaPO_4$) were found.[87] In addition, the presence of proteoglycans in the vicinity of the increased amount of abnormal elastin fibers was demonstrated by alcian blue or colloidal iron stain.

Finally, in early lesions, other specialized elastic tissue stains may be necessary for diagnosis.[92] In PXE skin lesions, several extra cellular matrix component alterations and deposition have been demonstrated[7,99]

On electron microscopy (EM) the first pathological sign of PXE is the calcification in elastic fibers that appear to be normal, in young patients in the lower dermis. In older ones most fibers show calcification and resulting degeneration.[36,54,73] That elastic fibers are the primary location of the calcification is

derived from the observation in decalcified endocardial lesions that decalcified elastic fibers had the same internal structure as adjacent non calcified fibers. The elastic fibers become pleomorphic, fragmented, and calcified. The extent of fragmentation of affected elastic fibers in PXE, is most dramatically related with disease progression.[94]

Initially, mineralization is seen as a central core of electron density by EM. As the elastic fibers become more and more mineralized, the central core becomes increasingly dense. Prior to fragmentation, the fiber will develop "holes" where the central portion of the core either disappears or spontaneously fades. Finally, the fibers become maximally calcified followed by fragmentation.

Two main kinds of calcifications have been described: one composed of hydroxyapatite and the other of $CaHPO_4$.[60] Other mineral precipitates, such as iron, phosphate, carbonate, and other ions have also been identified in altered elastic PXE fibers.[17,108,140,150] Furthermore, a thready material was found in the membrane as well as an increased amount of proteoglycans and glucosamine.[26,79,80] Studies in fibroblast culture from PXE patients provided evidence for increased degradation of sulfated proteoglycans and altered expression of extracellular components.[9,50,99,137] Moreover, matrix proteins, such as osteonectin, fibronectin, vitronectin, and fibrillin-2, with a high affinity for calcium ions, are uniquely associated with the altered elastic fibers in PXE.[7,8,98,139]

Recent light and EM miscroscopic studies of biopsies of PXE patients, healthy family members, and controls revealed that typical but relatively mild PXE symptoms occur in skin of heterozygous carriers. These changes were not present in one unaffected subject in the family.[10] In addition, in clinically normal skin of subjects suspected of PXE similar histological signs may also be found.[75] Finally, histological PXE-like changes have been described in traumatic scars from subjects with no PXE skin signs but with vascular abnormalities.[92] This seems to hold only for PXE patients and not for non-PXE cases with scar tissue, if only because elastic fibers take a long time to form and most scar tissue does not have much elastic fibers in it.

We do not consider the skin histology pathognomonic for PXE because calcific elastosis without perforations,[75,76] calciphylaxis after chronic renal failure,[96] as well as saltpeter and penicillamine intoxications, are histologically indistinguishable.[95] In elastosis perforans serpiginosum, the most distinctive perforating disorder of the skin, thickened elastic fibers that act as foreign bodies may be eliminated through the skin. Elastosis perforans serpiginosum occurs in PXE but also in several systemic disorders such as osteogenesis imperfecta, Down, Ehlers Danlos, as well as Marfan syndrome[73] and might be a source of confusion for the skin pathologists. Copper is essential to the formation of elastin. Because penicillamine, a copper chelating agent, induces elastosis perforans serpiginosum, it has been postulated that a disturbance in copper metabolism is at the basis of this disorder.[73] We might speculate whether this holds also for the pathogenesis of PXE. Interestingly, the skin in late onset focal dermal elastosis has a different histology.[77]

THE EYE

Elastic Components and Possible Function of Bruch's Membrane

BrM is an elastin- and collagen-rich membrane in the retina between the photoreceptor RPE and the choriocapillaris. BrM has no cell nuclei and its rather stable configuration over the first 40 years of life seems to be regulated by the adjacent RPE and choriocapillaris. BrM acts as an attachment site for the RPE cells and has a function in bidirectional transport of nutrients and metabolites between the RPE and the choriocapillaris.

Histologically, BrM can be divided into three to five layers, depending on to which structure the outer ones are attributed: the RPE basal lamina; an inner collageneous zone; a middle elastic layer; an outer collageneous zone; and a basement membrane of the endothelial cells of the choriocapillaris. The elastic fibers do not form a continuous layer; they are rather arranged in an interlacing network with spaces through which collagen bundles intermingle.[29] The co-localization of collagen types I, III, and VI in the elastic lamina suggests that the latter contributes to the integration of various extracellular matrix components into one functional unit.[5]

The mature elastic fibers and lamina in the extracellular matrix of BrM and other connective tissues provide elasticity and resilience to these tissues. Elastic fibers are synthesized during late prenatal and neonatal development. The turnover of elastin in normal adult tissues is quite low. Ultrastructurally, elastic fibers are complex structures composed of at least two morphologically distinguishable components, elastin and microfibrils.[85,116] The major component, elastin, has an unusual chemical composition. Elastin is rich in glycine, proline, and hydrophobic amino acids. The protein is synthesized via a soluble 72-kD precursor, tropoelastin, which is positioned on a microfibrillar scaffold before being cross-linked. The minor component of the elastic fiber, the microfibrils, consists of two forms of the glycoprotein fibrillin and two microfibril-associated

glycoproteins.[47,48,119,151] Numerous additional components are also thought to be present in the mature elastic fiber, such as lysyl oxidase, the elastin-binding protein (EBP), proteoglycans, osteopontin, emilin, fibulin-1, and other microfibril-associated proteins.[15,33,56,63,113,116]

Crucial to the proper function of the mature elastic fiber is the extensive extracellular crosslinking of tropoelastin at lysine residues. The crosslinking is preceded by selective lysine oxidation by the copper-requiring enzyme, lysyl oxidase.[31,56,149] The EBP, a 67-kD protein, binds tropoelastin in the endoplasmic reticulum and chaperones its secretion to assembly sites on the cell surface designated by cell-matrix receptors. The latter interacts again with microfibrillar proteins.[86] Microfibrils serve to align tropoelastin molecules in precise register so that crosslinking regions are juxtaposed prior to oxidation by lysyl oxidase.

Elastic fiber assembly thus is a highly complex process. It is evident that specific mutations in the genes encoding elastin or other proteins that are (in-)directly involved in elastic fiber assembly, will result in elastic fiber pathology and underlie heritable disease.[34,64,105]

Histopathology of Bruch's Membrane

The histopathologic features of AS were first described in 1892.[62] The changes in BrM are apparently similar to those noted in the skin.[60] Angioid streaks are ruptures in the thickened and calcified BrM.[36,55] The start of AS is marked by the discontinuities in the elastic layer of BrM in combination with loss of RPE pigment granules. In the next stage there are full-thickness breaks in BrM in combination with atrophy of the overlying RPE and photoreceptor cells plus ruptures of the underlying choriocapillaris.[36] Through calcification, BrM becomes brittle, which is considered to be the major factor leading to breaks in the membrane.[36] Iron deposits in AS in PXE may be due to precipitates in the calcium-rich environment or to old hemorrhages,[53] whereas AS associated with sickle cell disease seem to contain more ion and less calcium deposits due to hemolysis.[91] We presume that AS often have a similar phenotype with different pathogenesis, given the various hematological disorders associated with them. In Paget's disease there is calcification through all BrM, most marked in the posterior pole but extending till the ora serrata. In special segments the choriocapillaris had disappeared due to thickening of the capillary walls and probably obliteration of the lumen.[44]

Serial sections of one AS revealed, at one place, herniation of choroidal fibrillar collagen tissue and choriocapillaris into the break in BrM separating this membrane. This break was bridged by thinned, hypopigmented RPE and this explains together with the collagen tissue, the window defects and late staining of AS on fluorescein angiography.[44]

Clearly, the mechanisms of calcification of elastic fibers in Bruch's membrane and skin are equally complex and also incompletely understood, and they may be the result of abnormal elastic fiber development, alterations in the extracellular matrix, or degeneration of elastic fibers.[12,150] Surprisingly so far no histological data on peau d'orange or the comet-like tails have been encountered.

THE CARDIOVASCULAR SYSTEM

Calcification of the elastic layer of the small and medium-sized arteries has been demonstrated in patients with PXE[66,89] but was similar to routinely encountered atherosclerosis.[89] In muscular arteries, like the coronary or large peripheral ones, calcification begins in the internal and external elastic laminae, and later extends to the media and intima.[75] The endocardium shows characteristic intimal fibroelastic thickening and calcification of its elastic fibers[89] and this only rarely leads to complications[75] like restrictive cardiomyopathy. Specific pathologic anatomical descriptions of vessels in PXE patients could not be found in the literature.

Classification of PXE

From the previous sections describe, it may be clear that the diagnosis of PXE may be quite difficult. Neither the skin lesions nor the AS by themselves are pathognomonic. Von Kossa staining in skin biopsies will probably more often solve a diagnostic problem than demonstration of AS, because the differential diagnosis of elastin changes in a skin biopsy contains fewer disorders than the differential diagnosis of AS (Table 2). The comet-like tails in the retina seem to be pathognomonic for PXE but their prevalences are reported to be quite variable.

To make classification less cumbersome, a consensus PXE meeting was held in 1992 in which criteria for PXE diagnosis were laid down based on sensitivity and specificity of characteristic clinical and histological signs.[71] The attendees gave limited indications how they determined the sensitivity and specificity of the clinical signs. An initial consensus classification of five partially overlapping PXE categories was proposed, in which the cardiovascular signs were not included. It is to be expected that in the future finer elaborations in classification will be made.

Genetics of PXE

CLINICAL GENETICS

Mode of Inheritance

The majority of PXE patients are sporadic cases. In PXE families with a discernible mode of inheritance, AR inheritance is much more common than

AD segregation.[102–104,144] In PXE families, multiple affected siblings are common, but multigenerational transmission is rare. Initially, some investigators have attempted to subtype PXE based both on phenotypic expression and inheritance. Pope et al suggested, on the basis of the phenotype alone, that there are two AD and two AR forms of PXE.[103] A potential third autosomal-recessive subform, with an unusual combination of severe vision impairment and very mild skin lesions, was identified among 64 patients from South Africa and Zimbabwe.[147] Neldner et al concluded, on the basis of a 20-year follow-up study of 100 patients in the United States, that 90% of patients had arPXE.[94] The majority of patients of 52 Belgian families with PXE showed no family history for the disease. Without exception, all familial cases were of the ar PXE type.[30] Only one author suggested that ad PXE occurs in more than 10% of the cases.[102,103] Although true AD inheritance of PXE cannot be ruled out, at least part of the autosomal-dominant segregation in PXE pedigrees could possibly be explained by pseudo-dominant inheritance and manifestations of PXE in heterozygote carriers.[144]

Variability of Clinical Expression

Within and between PXE families, considerable variation in onset, progression, and severity of the disease exists.[127,141] In some patients, all three tissues—the skin, eye, and the cardiovascular system—are affected, whereas in others, even within the same family, only one or two tissues are involved.[39,127] Some cases have severe skin abnormalities, whereas others do not have clinically apparent skin lesions at all. In the latter group, the PXE diagnosis may only be established by biopsy of normal appearing flexural skin or from the middle portion of non-elevated 5 years or more old scars.[72] In six out of 10 subjects with PXE, scar tissue showed clumping and fragmentation of elastic fibers that was not present in scars from 10 non-PXE subjects; the normal-appearing flexural areas showed these abnormalities only in three of 10 PXE cases.[70] Interestingly, in some cases, the extent of clinical severity and the level of morphologic alteration in skin are not directly compatible.[116] Patients may have severe ophthalmologic or cardiac disease with little or no skin involvement, or vice versa.[30,61,70,127] The frequency of vascular symptoms also varies among reports. One study showed that claudication happened in one third of ar PXE cases.[94] In another study, no patients with ar PXE disease had claudication.[103]

In a number of arPXE families, carriers show subtle dermatological, ocular, or cardiovascular manifestations.[10,127] In general, the phenotypic variation in PXE appears to be unrelated to the mode of inheritance, the severity of the skin lesions, the visual problems, or the vascular complaints.[71] The high variability in clinical expression among and within PXE families may partly be due to a set of factors other than genetic background, such as nutritional, vitamins, hormones, life-style variables, and environmental factors.[94,109,142]

MOLECULAR GENETICS

Gene Location

In 1997, the PXE gene was localized to the human chromosome 16p13.1.[134,144] Given the results of the linkage studies, it was suggested that allelic heterogeneity in a single disease gene could account for both autosomal recessive and autosomal dominant forms of PXE. Subsequently, a multicenter collaborative effort genetically refined the locus to a region of about 820 kb region of 16p13.1.[68] In addition, a physical map spanning the obligate gene region was constructed, which facilitated the identification of the PXE disease gene.

Molecular Defects

The PXE gene: ABCC6

The progress on gene localization allowed rapid screening for mutations in candidate PXE genes in DNA of PXE patients. Recently, a gene from the obligate gene region on 16p13.1, ABCC6, was implicated in PXE.[12,69,111]

ABCC6 belongs to the ATP-binding cassette (ABC) gene sub-family C, together with ABCC1–12.[13,22,57,135] Members of this family are involved in a large variety of physiologic processes, such as signal transduction, protein secretion, drug and antibiotic resistance, as well as antigen presentation.[55] So far, the physiological function and the involvement of ABCC6 in the PXE phenotype remain unclear. However, the sequence and structural similarity of ABCC6 with ABCC1 and another recent study[58] suggest involvement of ABCC6 in transmembrane transport of polyanion-like substrates.[11,65] Other members of the ABC superfamily are involved in disorders such as Dubin-Johnson syndrome (ABCC2), cystic fibrosis (ABCC7), and familial persistent hyperinsulinemic hypoglycemia of infancy (ABCC8).[100,136,138]

ABCC6 consists of 31 exons spanning approximately 73 kb. The ABCC6 mRNA has a 4.5 kb open reading frame encoding a protein of 1,503 amino acids in length with a predicted molecular weight of 165 kD.[11,65] This protein is the multidrug resistance protein 6 (MRP6). MRP6 is composed of three hydrophobic membrane spanning domains, 17 transmembrane spanning helices and two evolutionary

conserved ATP- binding folds (NBFs) (Fig. 4).[55] Mutation analysis of a number of ABC proteins indicates that the latter two regions are critical for ATPase and thus for ATP driven transport functions.[13,124] The proposed location of the NH2 terminus is extracellular. Recent findings indicate that at least two pseudogenes of ABCC6 in the human genome exist. The reiterated parts are homologous to the 5□of ABCC6, from exon 1 to 4 and from exon 1 to 9.[16,107]

Very high expression of the ABCC6 mRNA was found in human kidney and liver.[65] In tissues frequently affected by PXE, including skin, vessel wall, and retina, the expression of ABCC6 was lower.[12] In other tissues, apparently not involved in PXE, such as bladder, brain, heart, ovary, salivary gland, spleen, stomach, testis, thyroid gland, and tonsil, the expression of ABCC6 is also low.[65] Interestingly, no expression of ABCC6 was found in an elastin-rich tissue like the lungs.[11,65]

Identified Mutations

Simultaneously, two research groups identified mutations in the ABCC6 gene causing sporadic PXE, ar PXE and ad PXE.[12,69] Subsequently, these findings were confirmed by several groups.[45,67,88,111,112,133] To date, 49 mutations and subchromosomal deletions were identified by screening 170 patients or families (Table 3). Mutations have been identified in 18 out of 31 exons and in two introns of ABCC6. Of the 49 mutations, there are 8 nonsense, 25 missense, and 13 frameshift mutations. In addition, three intragenic deletions spanning nine exons of ABCC6 as well as one deletion completely removing the ABCC1, ABCC6 and MYH11 genes were found. The majority of mutations introduce frame shifts and stop codons that lead to premature terminations or shorter proteins.

So far, the most common mutations reside in the second half of the gene, particularly, in exon 24 and exon 28 (Fig. 4). Exon 24 encodes the putative eighth intracellular loop, which is probably essential for normal ABCC6 function. The R1141X mutation in exon 24 was reported by four research groups and appears to be a common mutation underlying PXE. R1141X generates a stop codon at cDNA position 3421 and presumably results in a reduction in mutant mRNA levels by nonsense-mediated RNA decay.[25,69] Mutations appear to be also frequent in exons 28–30, corresponding to the NBF2 region, where the function of the protein might be abolished by the change in a single amino acid. Several other mutations were found within exon 16, 21, 24, 27, 28, and 30 of ABCC6, located in highly conserved coding domains of ABCC6 (Fig. 4). The detailed function of most of these variants remains to be elucidated upon development of functional assays for PXE.

Transport Function of ABCC6 in Relation to PXE

At present, the relationship between biomolecules transported by ABCC6 and the PXE disease phenotype is not clear. Given the high expression of ABCC6 in kidney and liver, it is possible that PXE is in fact a heritable systemic disorder.[142] In this scenario, a primary defect of ABCC6 in liver and kidney could result in abnormal levels of ABCC6 substrates in the blood, which could affect the elastic fiber assembly at specific sites in the body. On the other hand, (lower) ABCC6 expression also has been observed in tissues affected by PXE.[12] Consequently, it is also possible that local ABCC6 defects at multiple sites in the body result in a PXE phenotype. Finally, it may be possible that the PXE phenotype is caused by an indirect cumulative effect of both systemic and local ABCC6 defects.

SYSTEMIC TRANSPORT FUNCTION OF ABCC6

The exact physiological role of ABCC6 is not yet known. Functional investigations showed a high level of ABCC6 expression in excretory organs such as liver and kidney. The presence of ABCC6 in the latter tissues could be compatible with a role in cellular detoxification as a drug transporter, glutathione (GSH) conjugate (GS-X) pump or multispecific organic anion transporter as ABCC1.[32] However, no indications for involvement of ABCC6 in drug resistance were obtained.[65]

The rat homolog of human ABCC6 or mrp6 demonstrates a 79% amino acid similarity with human ABCC6.[65] Both mrp6 and ABCC6 exhibit a similar tissue distribution, with the highest expression in the liver, followed by kidney.[65,81] The localization of mrp6 was found at the lateral and, to a lesser extent, at the canalicular plasma membrane of rat hepatocytes using a polyclonal antiserum raised against a C-terminal peptide.[81] Initially, transport studies showed that none of the tested classical substrates such as glutathione-, glucuronide-, and sulfate-conjugates were transported by rat mrp6. Recent experiments showed that human ABCC6 transports organic anions such as glutathione conjugates.[58] In vitro, the endothelin receptor antagonist BQ-123 is transported by mrp6.[81] Our results show that ABCC6 in human liver is also located at the basolateral membrane of hepatocytes.[123] Such a distribution suggests that ABCC6 may potentially pump organic anions or other substrates from the liver back into the blood. How this eventually could result in elastin or elastic fiber accumulation in Bruch's membrane remains unclear. Further clarification of the transport function of mrp6 will help to elucidate the function of human ABCC6 and the etiology of PXE.

TABLE 3

Summary of ABCC6 Mutations in PXE Patients

Mutation	Protein Alteration	Nucleotide Substitution	Location	Reference
Nonsense				
	Q378X	1132C > T	Exon 9	16,107
	R518X	1552C > T	Exon 12	88
	Y768X	2304C > A	Exon 18	67
	R1030X	3088C > T	Exon 23	67
	R1141X	3421C > T	Exon 24	12,45,67,107,111,112,133
	R1164X	3490C > T	Exon 24	88,112
	Q1237X	3709C > T	Exon 26	67
	R1398X	4192C > T	Exon 29	67
Missense				
	T364R	1091C > G	Exon 9	107
	N411K	1233T > G	Exon 10	67
	A455P	1363G > C	Exon 11	142
	R518Q	1553G > A	Exon 12	67,142
	F568S	1703T > C	Exon 13	67
	L673P	2018T > C	Exon 16	67
	R765Q	2294G > A	Exon 18	67
	R1114P	3341G > C	Exon 24	67
	S1121W	3362C > G	Exon 24	67
	R1138W	3412C > T	Exon 24	111
	R1138Q	3413G > A	Exon 24	67,111
	R1138P	3413G > C	Exon 24	67
	G1203D	3608G > A	Exon 25	67
	V1298F	3892G > T	Exon 28	67
	T13011	3902C > T	Exon 28	67
	G1302R	3904G > A	Exon 28	67
	A1303P	3907G > C	Exon 28	67
	R1314W	3940C > T	Exon 28	67
	R1314Q	3941G > A	Exon 28	67
	G1321S	3961G > A	Exon 28	67
	R1339C	4015C > T	Exon 28	67,133
	Q1347H	4041G > C	Exon 28	67
	G1354R	4060G > C	Exon 29	107,142
	D1361N	4081G > A	Exon 29	67
	11424T	4271T > C	Exon 30	67
Frameshift				
Splicing		IVS21 + 1G >T	Intron 21	67,142
		IVS26−1G >A	Intron 26	67,111,112
Deletion		179del9	Exon 2	107
		179-195del	Exon 2	67
		960delC	Exon 8	88
		1944del22	Exon 16	12
		1995delG	Exon 16	67
		2322delC	Exon 18	67
		2542delG	Exon 19	67
		3775delT	Exon 27	12,67
		4101delC	Exon 29	67
Insertion		938-939insT	Exon 8	67
		4220insAGAA	Exon 30	12
Intragenic deletion			Exon 23-29	67,112
			Exon 15	67
Intergenic deletion			ABCC6	12,88

LOCAL RETINAL TRANSPORT FUNCTION OF ABCC6

ABCC6 Expression in the Retina

Bergen et al detected ABCC6 expression in various tissues in man.[12] Low expression levels of ABCC6 were observed in the retina as well as other tissues usually affected by PXE, including skin and vessel wall. This may suggest that ABCC6 might regulate the extracellular matrix movement between the inner retina and the photoreceptor-RPE-Bruch's membrane complex. The ABCC6 deficiencies might

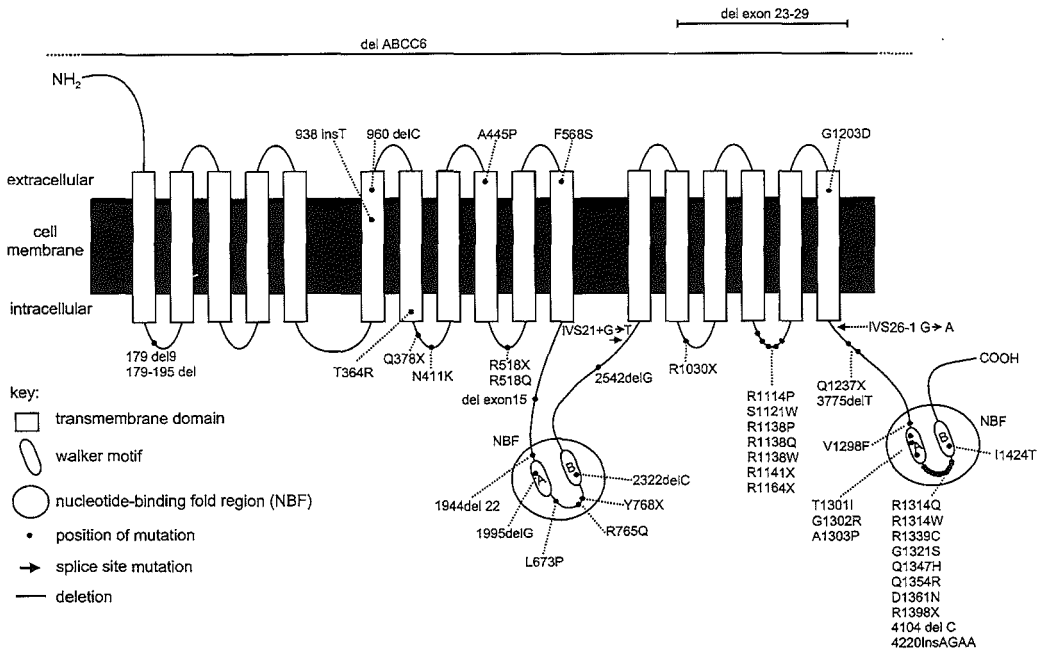

Fig. 4. Location of the mutations causing pseudoxanthoma elasticum in ABCC6 with respect to the proposed topological model of the ABCC6 including the three membrane-spanning domains, 17 transmembrane spanning helices, and two nucleotide binding folds.

lead to aberrant transport mechanisms in retina and result indirectly in abnormal elastin or elastic fiber accumulation in the RPE/Bruch's membrane secondary to fiber assembly.

Transport of Biomolecules in the Retina

The outer retina, including the photoreceptors and RPE, has a high level of metabolic activity.[35] A large number of biomolecules as well as organic molecules are transported through the retina during the visual process. Common molecules involved in the various functions of retinal tissues include retinol, rhodopsin, rim protein, guanylate cyclase activating protein, RPE-specific protein, cellular retinaldehyde-binding protein, interphotoreceptor retinoid-binding protein, and cellular retinoid-binding protein.[51,145] In addition, the retina plays a role in regulating the balance of local ionic and nutrient concentrations. Glucose, glutathione, and oxygen are required for generation of electrical activity. Glucose consumption, lactic acid production and oxygen utilization suggest that there is a CO2/bicarbonate buffer system in the retina. Transport of glucose in the retina is regulated by the extracellular concentration of glucose. Despite glucose metabolism, the abundance of substrates for energy (ATP) stores, such as glutamate, glutamic acid, malate, and succinate can also be metabolized in the retina.[51] In principle, one or more of these components could be transported by ABCC6 in the retina.

The Role of the Photoreceptor-RPE Complex in Retinal Transport

In the normal eye, the RPE forms a confluent monolayer of polarized cells located between the retinal photoreceptors on one side, and BrM and capillaries of the choroid on the other side. The apices of the photoreceptor outer segments interact with the RPE. The basal surface of the RPE adheres to BrM. Photoreceptors form one functional complex with the RPE cells and the extracellular matrix structure of BrM. The complex has a high metabolic activity and receives most of its blood supply from the choroid.

In addition to collagenous and elastic components, BrM contains a variety of proteoglycans, such as heparan, dermatan, and chondroitin sulphate-types.[51,78,84] Histological studies suggest the presence of anionic sites, probably consisting of sulphate proteoglycans, in BrM. These anionic sites are probably charge barriers, which have both structural and filtration properties. The latter suggests that BrM may

retard the movement of anionic molecules from the choriocapillaris to the RPE and outer neural retina or vice versa.

The RPE is a polarized cell layer with tight junctions. The transport of biomolecules, retinoids, and glucose through the RPE is bidirectional between retina and choriocapillaris. Throughout life waste material is discharged from the RPE onto BrM. The waste material is cleared toward the choroid. Alterations in the composition of photoreceptor-RPE-BrM complex by defective ABCC6 mediated active transport of anorganic anions could affect the transport properties and the distribution of molecules, and ultimately, impair visual function.

Future Prospects

The recent progress in the identification of the gene for PXE is a significant step. New insights in the etiology of the disease have opened up new research avenues, but many questions remain. The functional consequences of mutations in ABCC6 gene are not yet understood. It is essential to obtain additional genetic mutation and phenotypic data for a complete overview of all mutations that lead to PXE. With these data, further insight will be gained in the genotype–phenotype relationship and the etiology of PXE. Furthermore, these data will help to direct and improve the service that clinical geneticists can offer to the patients.

To unravel the pathogenesis of PXE, additional studies should be focused on the identification of the molecules transported by ABCC6 and elucidation of the putative role of these substrates in the mineralization of elastic fibers in BrM and elsewhere in the body. Among others, animal models, such as mice lacking mrp6, may be a helpful tool in the elucidation of the molecular mechanisms underlying PXE.

Method of Literature Search

Medline, PubMed, and OVID search of relevant literature spanning the period 1966 to January 2002 was performed. Search terms were the following: *ATP-binding cassette transporter, (autosomal dominant) angioid streaks, Bruch's membrane, classification, differential diagnosis, elastic fibers, hereditary angioid streaks, pseudoxanthoma elasticum, photoreceptor, retina, and retinal pigment epithelium.* Additional references included standard textbooks on biology and biochemistry of the eye as well as on dermato-pathology. Articles were also obtained from the reference lists of other articles.

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Chapter 2

ABCC6/MRP6 Mutations: Further Insight in the Molecular Pathology of Pseudoxanthoma Elasticum.

ARTICLE

ABCC6/MRP6 mutations: further insight into the molecular pathology of pseudoxanthoma elasticum

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Pseudoxanthoma elasticum (PXE) is a hereditary disease characterized by progressive dystrophic mineralization of the elastic fibres. PXE patients frequently present with skin lesions and visual acuity loss. Recently, we and others showed that PXE is caused by mutations in the ABCC6/MRP6 gene. However, the molecular pathology of PXE is complicated by yet unknown factors causing the variable clinical expression of the disease. In addition, the presence of ABCC6/MRP6 pseudogenes and multiple ABCC6/MRP6-associated deletions complicate interpretation of molecular genetic studies. In this study, we present the mutation spectrum of ABCC6/MRP6 in 59 PXE patients from the Netherlands. We detected 17 different mutations in 65 alleles. The majority of mutations occurred in the NBF1 (nucleotide binding fold) domain, in the eighth cytoplasmatic loop between the 15th and 16th transmembrane regions, and in NBF2 of the predicted ABCC6/MRP6 protein. The R1141X mutation was by far the most common mutation identified in 19 (32.2%) patients. The second most frequent mutation, an intragenic deletion from exon 23 to exon 29 in ABCC6/MRP6, was detected in 11 (18.6%) of the patients. Our data include 11 novel ABCC6/MRP6 mutations, as well as additional segregation data relevant to the molecular pathology of PXE in a limited number of patients and families. The consequences of our data for the molecular pathology of PXE are discussed.

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Introduction

Pseudoxanthoma elasticum (PXE) is a hereditary disorder of the connective tissue. The disease is characterized by dystrophic mineralization of elastic fibres of the skin, retina, and cardiovascular system.[1–3] Patients frequently have dermal lesions, experience progressive loss of visual acuity, and are at increased risk for cardiovascular complications. The clinical expression of PXE in patients is highly variable,[4,5] and the mode of inheritance of PXE are currently not completely understood. The majority of PXE patients are sporadic cases. In a large subset of families, PXE segregates in an autosomal recessive (ar) fashion.[6] In a small number of families, autosomal dominant (ad) inheritance has been reported.[6,7]

We and others previously localized the PXE gene to chromosome 16p13.1,[8,9] and found that mutations in the *ABCC6/MRP6* (*MRP6*) gene are associated with all genetic forms of PXE.[10–12] The *ABCC6/MRP6* gene is a member of the ATP-binding cassette (ABC) family, and encodes a transport protein of 1503 amino acids.[13,14] The gene

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contains Walker A and B motifs typical for ABC proteins and 17 transmembrane domains.[15] High ABCC6/MRP6 mRNA expression levels were found in kidney and liver, while lower expression was found in tissues usually affected by the disease.[10,16] Using monoclonal antibodies, we recently localized human *ABCC6/MRP6* to the basolateral side of hepatocytes and the proximal tubules of kidney.[17] Recently, Ilias *et al* (2002) found that glutathione conjugates, including leukotriene-C4 (LTC_4) and N-ethylmaleimide *S*-glutathione (NEM-GS), are actively transported by human *ABCC6/MRP6*. In three ABCC6/MRP6 mutant forms, loss of *ABCC6/MRP6* transport activity appears to be directly responsible for PXE.[18]

Interpretation of the results from mutational analysis of ABCC6/MRP6 is complicated by the presence of two ABCC6/MRP6 pseudogenes in the genome and the multiple presence of larger and smaller deletions spanning (part of) the gene.[10,19–22] In addition, still unknown molecular or environmental factors that could influence the variable clinical expression of the disease in patients complicate the correct assessment of genotype–phenotype relations. In this study, we present and discuss the results of ABCC6/MRP6 mutation analysis in 59 PXE patients and families from The Netherlands.

Results

Summary of mutational screening

The ABCC6/MRP6 gene was screened in 59 patients from apparently unrelated Dutch patients and families with PXE. In 41.9% of the families, PXE segregated in a clearcut ar fashion. A large proportion of patients (27.9%) were sporadic cases. In 30.2% of the families, the segregation pattern was not clear or revealed a putative dominant inheritance pattern (A Plomp, personal communication). Using PCR, SSCP, and direct sequencing, our ABCC6/MRP6 mutation detection rate was 55.1% (total number of mutations found divided by the total number of alleles). We detected at least one disease-causing allele in 43 patients (72.9%). We found 17 different ABCC6/MRP6 mutations that were assigned to 65 alleles (Table 1). A variety of mutations were observed including nonsense, missense, putative splice site mutations as well as deletions and one insertion. In our patient cohort, mutant alleles occurred in all combinations including homozygous, heterozygous, compound heterozygous, and hemizygous forms. A total of 11 different mutations were apparently unique to our patient group and have not been described by others. Combining our data with those of the literature, we conclude that 57 different ABCC6/MRP6 mutations are now known to cause PXE (Table 2).

Table 1 Summary of ABCC6/MRP6 mutations found in our cohort of 62 PXE patients from the Netherlands and summary of functional consequences

No. of patients	*Allele 1*	*Consequence*	*Exon*	*Allele 2*	*Consequence*	*Exon*	*Mode of inheritance in family*
1	2247C>T	Q749X	17				s
1	3421C>T	R1141X	24	2247C>T	Q749X	17	ar
9	3421C>T	R1141X	24				ar,s, n
1	3421C>T	R1141X	24	1944del22	Frameshift	16	n
3	3421C>T	R1141X	24	Deletion	A995del405	23–29	ar
1	3421C>T	R1141X	24	4182delG	Frameshift	29	ar
1	3421C>T	R1141X	24	3775delT	Frameshift	27	s
3	3421C>T	R1141X	24	3421C>T	R1141X	24	ar, s
1	2294G>A	R765Q	18	3775delT	Frameshift	27	ar
1	3341G>A	R1114H	24				n
1	3390C>T	T1130M	24	3390C>T	T1130M	24	ar
1	3663C>T	R1221C	26	3775delT	Frameshift	27	n
1	3904G>C	G1302R	28				s
1	3907G>A	A1303P	28	Deletion	A995del405	23–29	ar
1	4182G>T	K1394N	29	Deletion	A995del405	23–29	ar
1	4182delG	Frameshift	29				n
1	4182delG	Frameshift	29	4182delG	Frameshift	29	ar
1	4377C>T	R1459C	30				ad?, s,n
2	3775delT	Frameshift	27				s,n
1	3775delT	Frameshift	27	Deletion		all?	ar
1	3775delT	Frameshift	27	3775delT	Frameshift	27	ar
1	4220insAGAA	Frameshift	30				n
1	IVS17-12delTT	?	Intron17				n
1	1944del22	Frameshift	16				n
2	Deletion	A995del405	23–29				n
1	Deletion	A995del405	23–29	Deletion		all[a]	ar
3	Deletion	A995del405	23–29	Deletion	A995del405	23–29	ar

S = sporadic, ar = autosomal recessive, ad = autosomal dominant, n = not known. ? indicates that the (potential) mutation observed is not characterized in detail yet and requires further study. [a]indicates a large deletion spanning the ABCC6/MRP6, ABCC1, and MYH11 genes. The exact breakpoints of this deletion, and another large deletion (indicated by deletion all?) are not characterized in detail yet.

Table 2 Summary of ABCC6/MRP6 mutations associated with PXE known today: our data combined with those of the literature

Mutation	*Protein alteration*	*Nucleotide substitution*	*Location*	*Reference*
Nonsense	Q378X	1132C > T	Exon 9	19,20
	R518X	1552C > T	Exon 2	41
	Q749X	2247C > T	Exon 17	This study
	Y768X	2304C > A	Exon 18	22
	R1030X	3088C > T	Exon 23	22
	R1141X	3421C > T	Exon 24	12,20,22,38,39, this study
	R1164X	3490C > T	Exon 24	12,41
	Q1237X	3709C > T	Exon 26	22
	R1398X	4192C >T	Exon 29	22
	T364R			
Missense	N411K	1091C > G	Exon 9	20
	A455P	1233T > G	Exon 10	22
	R518Q	1363G > C	Exon 11	38
	F568S	1553G > A	Exon 12	22,38
	L673P	1703T > C	Exon 13	22
	R765Q	2018T > C	Exon 16	22
	R1114P	2294G > A	Exon 18	22, this study
	R1114H	3341G > C	Exon 24	22
	S1121W	3341G > A	Exon 24	This study
	T1130M	3362C > G	Exon 24	22
	R1138W	3390C > T	Exon 24	This study
	R1138Q	3412C > T	Exon 24	12
	R1138P	3413G > A	Exon 24	12,22
	G1203D	3413G > C	Exon 24	22
	R1221C	3608G > A	Exon 25	22
	V1298F	3663C > T	Exon 26	This study
	T1301I	3892G > T	Exon 28	22
	G1302R	3902C > T	Exon 28	22
	A1303P	3904G > A	Exon 28	22, this study
	R1314W	3907G > C	Exon 28	22, this study
	R1314Q	3940C > T	Exon 28	22
	G1321S	3941G > A	Exon 28	22
	R1339C	3961G > A	Exon 28	22
	Q1347H	4015C > T	Exon 28	22,39
	G1354R	4041G > C	Exon 28	22
	D1361N	4060G > C	Exon 29	20,38
	K1394N	4081G > A	Exon 29	22
	I1424T	4182G > T	Exon 29	This study
	R1459C	4271T > C	Exon 30	22
		4377C > T	Exon 30	This study
Frameshift		IVS17-12delTT	Intron 17	This study
		IVS21+1G>T	Intron 21	22,38
		IVS26-1G>A	Intron 26	12,21,22
		179del 9	Exon 2	20
		179-195del	Exon 2	22
		960del C	Exon 8	41
		1944del22	Exon 16	This study
		1995delG	Exon 16	22
		2322delC	Exon 18	22
		2542delG	Exon 19	41
		3775delT	Exon 27	This study
		4104delC	Exon 29	22
		4182delG	Exon 29	This study
		938-939insT	Exon 8	22
		4220insAGAA	Exon 30	This study
Large deletion			Exons 23–29	21, This study
			Exon 15	22
			ABCC1, ABCC6	41, this study

Mutation types

The mutation types found in this study are summarized in Table 1. We observed two distinct nonsense mutations, R1141X and Q749X in 24 out of 117 alleles (20.5%). R1141X occurred in 22/117 alleles (18.8%) and was found in a homozygous, heterozygous, or compound heterozygous form in 19 patients (32.2%). This mutation was the most frequent ABCC6/MRP6 mutation found in our patient cohort. The Q749X nonsense mutation occurred in only two PXE patients in heterozygous or compound heterozygous form.

We found eight different missense mutations (R765Q, R1114H, T1130M, R1221C, A1303P, G1302R, K1394N, R1459C) that occurred in various combinations in nine alleles of eight patients. In addition, we detected five different intragenic ABCC6/MRP6 deletions. The first one was a 22 base pair(bp) deletion at position 1944 of the cDNA in exon 16. This deletion occurred in heterozygous and compound heterozygous form in two patients, and results in a shorter mRNA chain, and the introduction of a stop codon at cDNA position 2064. The second one was a deletion of a T at cDNA position 3775 in exon 27, which results in a frameshift at codon 1259 and premature termination at codon 1272. This mutation was detected in five alleles of three patients. The third deletion was a deletion of a G at position 4182, the last nucleotide of codon 1394 in exon 29, which changes the codon for Leu at 1402 into a stop codon. This deletion occurred in six alleles of five patients. The last deletion we observed spans exons 23–29, which is predicted to result in a 405 amino-acid deletion in the polypeptide. The latter deletion was found on 13 alleles (11.1%) of 11 patients (18.6%) and was the second most frequent mutation in this study after R1141X.

One allele with a large deletion encompassing the entire ABCC6/MRP6, ABCC1 and MYH11 genes was found in one patient. Apart from the five intragenic deletions, a putative splice site deletion mutation (IVS17-12 delTT) was found.

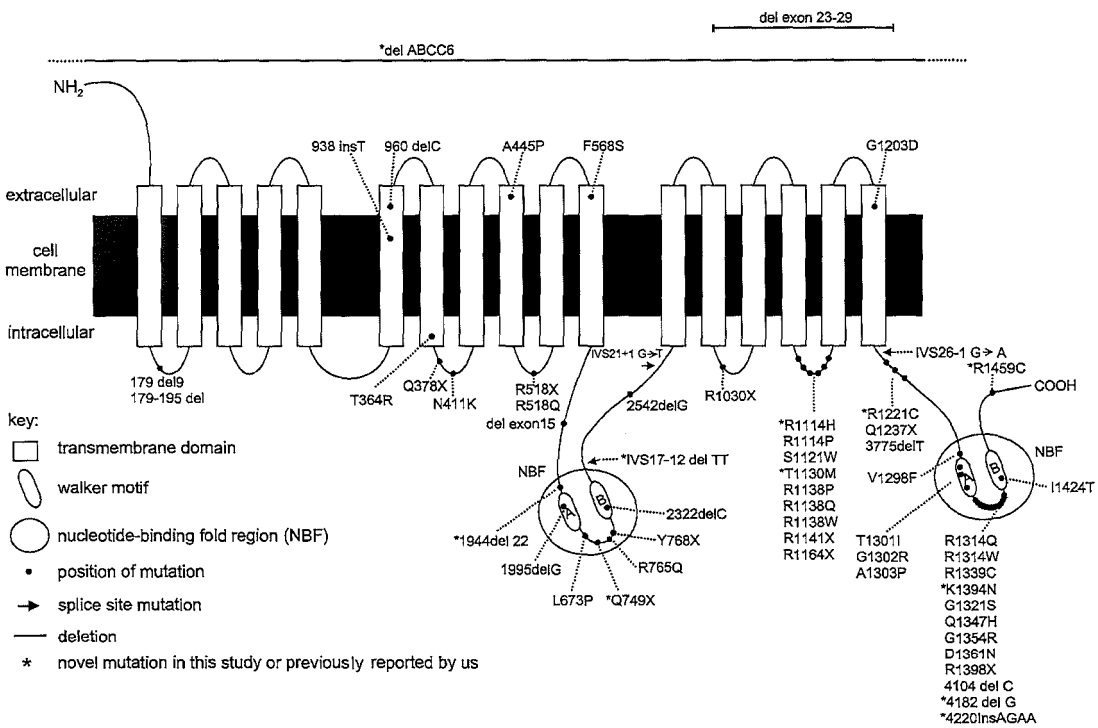

Figure 1 A schematic representation of the MRP6 protein with 17 transmembrane domains is shown. Note that the glycosylated NH_2 terminus is located outside the cell. The carboxy terminus is located inside the cell. The two ATP-binding domains are indicated with two black circles. The mutation spectrum in ABCC6/MRP6 associated with PXE is shown. Novel mutations presented in this study, or previously reported by us, are marked with asterisks.

The only insertion detected was a 4 bp insertion at position 4220, codon 1047, which alters the reading-frame, and most likely abolishes protein function.

In 20 PXE patients, we only found a single ABCC6/MRP6 mutation in one allele, but no nonsynonymous sequence changes in the second allele.

Mutation spectrum and distribution

The summary of our data, and our data combined with those of the literature are presented in Figure 1 and Table 2. Our data indicate that the diversity among the ABCC6/MRP6 nonsense mutations and deletions in our patient cohort was relatively low. However, the few nonsense mutations, which we did identify, occurred relatively frequently. In contrast, the diversity among our missense mutations was large, while the frequency of each missense mutation was relatively low.

The mutations we found in our cohort occurred all in cytoplasmatic domains of the ABCC6/MRP6 toward the carboxy-terminal end of the protein, within or beyond the first NBF1 domain. We detected three clusters of mutations in the predicted ABCC6/MRP6 protein: in the NBF1 domain, in the 8th cytoplasmatic loop between the 15th and 16th transmembrane regions, and in NBF2 (see Table 1 and Figure 1). In the NBF1 domain, we found three different NBF1-specific mutations in five alleles (4.3%). In the second, cytoplasmatic-loop cluster of mutations, we detected mutations in 25 alleles (21.4%) of 21 patients (excluding large deletions spanning this domain). The R1141X mutation, the most common PXE mutation found in our cohort, is located in this domain. Five different NBF2-specific mutations were found on nine alleles (7.7%) from seven patients. Outside these three domains, relatively infrequent and widely distributed mutations are found corresponding to 11 alleles of nine PXE patients. Indeed, the mutation distribution in our cohort reflects those in other studies if we combine all ABCC6/MRP6 mutation data known to date: Approximately 80% of the mutations occur in cytoplasmatic domains; NBF1 is mutated on eight ABCC6/MRP6 alleles (2.3%), the eighth cytoplasmatic domain on 79 alleles (22.3%), and NBF2 on 41 alleles (11.6%).

Segregation of PXE in pedigrees

To illustrate the complex genetics of PXE, we studied a number of patients and pedigrees from our cohort in more detail (Figure 2). The clinical features of these patients are presented in Table 3.

The PXE patient from pedigree PXE26101 was initially classified as a 'sporadic' patient. Molecular analysis revealed that she was homozygous for the ABCC6/MRP6 R1141X mutation. Both her healthy parents were carriers of this mutation, which is compatible with normal ar inheritance. Using RT-PCR and direct sequencing, we found that this mutation was also present in

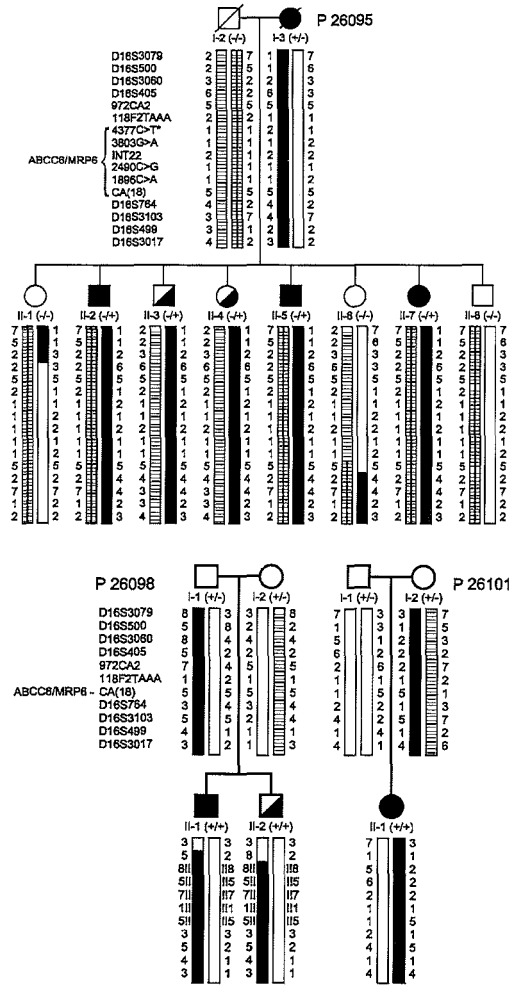

Figure 2 Pedigrees of three Dutch families and haplotypes of microsatellite markers within or flanking the ABCC6/MRP6 gene. Black squares and circles denote severely affected individuals and half-black/half-white symbols mildly affected individuals. Exact description of the phenotypes are presented in Table 3. The bars denote haplotypes segregating with the PXE phenotype. The ABCC6/MRP6 gene is located in between the markers D16S764 and 118F2TAA; – indicates the absence of the mutation and + the presence of the mutation. In PXE patients of pedigree P 26095, detailed DNA and RNA analyses revealed one allele with a mutation (R1495C) as well as a wild-type allele. The R1495C mutation segregates through the maternal line, since a nephew of the mother carries the mutation also. Note that in pedigree P26098, for a number of markers (indicated with !!), no Mendelian inheritance is observed, which suggests the presence of a submicroscopic deletion. Indeed, further molecular analysis indicated that this deletion spans the ABCC6/MRP6, ABCC1, and MYH1 genes.

Table 3 Clinical characteristics of patients from the pedigrees described in Figure 2

Pedigree	*Family member*	*Age*	*Age of onset*	*Skin*	*Biopsy*	*Eyes*	*Cardiovascular*	*Genotype* *Allele 1*	*Allele 2*
26101	II-1	44	12	+	d	AS	ht,TIA	R1141X	R1141X
	I-1	69		n	d	n	MI	R1141X	WT
	I-2	69		n	d	n	Chest pain	R1141X	WT
26098	II-1	46	22	+	d	AS,MD	GI hemorrhage	delABCC6	del exon 23–29
	II-2	40		n	d	AS	n	delABCC6	del exon 23–29
	I-1	71		n	±	Drusen	Multiple CI	delABCC6	WT
	I-2	73		d	d	d	MI	del exon 23–29	WT
26095	I-3	83	52	+	+	AS,MD	ht	d	
	II-1	66		n	d	n	n	WT	WT
	II-2	63	48	+	d	MD	n	R1459C	WT
	II-3	61	61	±	±	AS	n	R1459C	WT
	II-4	59		n	n	AS,PdO	n	R1459C	WT
	II-5	57	55	+	d	AS,neo,PdO	n	R1459C	WT
	II-6	56		n	d	n	n	WT	WT
	II-7	54	49	+	d	AS,neo	n	R1459C	WT
	II-8	52		n	d	n	n	WT	WT

AS = angioid streaks; CI = cerebral infarct; GI = gastrointestinal; ht = hypertension; MD = macula degeneration; MI = myocardial infarct; n = normal; neo = neovascularization; PdO = peau d'orange; RD = retinal detachment; TIA = transient ischaemic attack; WT = wild type; + = affected; ± = possibly affected; d = not tested.

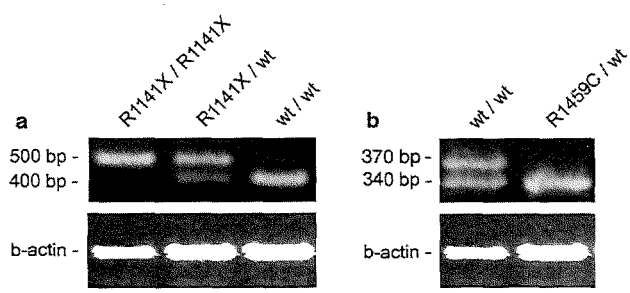

Figure 3 RT-PCR analysis of ABCC6 expression in leukocytes in individuals homozygous and heterozygous for the R114X mutation and in individuals heterozygous for the R1459C mutation compared with wild-type ABCC6. RT-PCR primers and conditions are given in the Material and methods section. (a) A wild-type cDNA fragment (wt) from the RT-PCR reaction is cut with *BsiY*1 in fragments of 400 and 100 bp. The R1141X mutant allele leads to loss of a *BsiY*I restriction site; the mutated allele is therefore not cut and presents with a 500 bp band. (b) The R1495C mutation abolishes a *AciI* restriction site in the cDNA: Wild type sequences are cut and result in *AciI* fragments of 310 and 30 bp; R1495C mutated fragments result in a single *AciI* fragment of 340 bp. All findings (a and b) were confirmed by direct sequencing.

ABCC6/MRP6 mRNA isolated from the patients' peripheral blood.

We previously reported on a sporadic patient with a large DNA deletion spanning the ABCC6/MRP6, ABCC1, and MYH11 genes on the maternal allele.[10] We initially suggested that PXE in this patient (II-1 in pedigree PXE26098, also called P-06) could be caused by haploinsufficiency at the ABCC6/MRP6 locus, given the apparent absence of a mutation in the second allele. However, an intragenic deletion spanning exons 23–29 on the paternal allele initially escaped our attention. Molecular analysis of the breakpoints of this latter deletion showed that it was similar to the exons 23–29 deletion recently reported by Ringpfeil *et al.*[21] This intragenic deletion in the family was confirmed to be present in mRNA from leukocytes. Consequently, PXE in this (compound heterozygous deletion) patient is most likely caused by complete loss of function of ABCC6/MRP6. It is remarkable that the patient (II-1) was severely affected, while his brother, who had the same genotype for the ABCC6/MRP6 locus, has only asymptomatic angioid streaks.

In a putative ad PXE family, molecular analysis was carried out (PXE 26095). The family consisted of an affected mother, a healthy father, three severely affected, two mildly affected,

and three nonaffected offspring. The characteristics of the phenotype are given in Table 3. We detected an ABCC6/MRP6 missense mutation (R1459C) heterozygously present in the DNA and RNA of all affected individuals.

We were not able to find a mutation or deletion on the second allele in the patients' DNA. Normal segregation and heterozygosity of multiple polymorphic markers intragenic and flanking ABCC6/MRP6 markers do not suggest the presence of a large deletion extending into ABCC6/MRP6 either. Restriction analysis of an RT-PCR product of exon 30 revealed the presence of the R1459C mutation in one transcript, while the other transcript was wild type (Figure 3). Sequencing of the entire ABCC6 cDNA in two patients (II-2, II-7) through RT-PCR of RNA from peripheral blood showed the presence of a mutated (R1459C), as well as an entirely normal, wild-type ABCC6 transcript, without mutations (not shown).

We found the R1459C mutation also in the DNA of a maternal nephew, who, unfortunately, refused clinical examination (not shown). However, the latter finding provides further evidence that the R1459C mutation in this pedigree segregates via the maternal line to the affected children. No consanguinity between the parents was found for four generations back.

Discussion

PXE mutations – Functional consequences

Although our mutation detection rate (55.1%) is comparable to another study of smaller European cohorts,[22] it remains likely that we still did not detect a substantial amount of mutations. This is probably because of a number of reasons. First, it is well known that the sensitivity of SSCP is only 70–80%. Consequently, 20–30% of the mutations will not be detected. Second, a number of disease-causing mutations may still be present in those promotor or intron regions that we did not screen (yet). In addition, we may have missed mutations in the ABCC6/MRP6 promotor region given its homology with ABCC6/MRP6-ψ2 pseudogene sequences. Finally, we may have missed still intragenic deletions using Southern analysis because of crosshybridization with putative pseudogene sequences.

An alternative explanation of our results may be that other, not directly ABCC6/MRP6-related genes or genetic or environmental factors are involved in the expression of the PXE phenotype or that rare ABCC6/MRP6 mutations cause a dominant PXE phenotype. If the latter is the case, a yet-to-be defined number of individuals with an ABCC6/MRP6 mutation in a single allele, may actually present with (almost) the complete disease phenotype.

The distribution of PXE-associated mutations in ABCC6/MRP6 is unequal. The majority of the mutations occurred toward the carboxy-terminal end and in the cytoplasmatic domains of the protein. This phenomenon can be partly explained by the intracellular and carboxyterminal location of the evolutionary conserved NBF1 and NBF2 domains, known to be essential for ATP-driven transport of the ABC-protein family. Indeed, the clustering of mutations at the NBF1 and NBF2 domains of ABCC6/MRP6 indicates that these two regions are essential for the normal function of this transport protein. For NBF1, the latter was recently demonstrated by transport activity studies.[18]

In order to obtain further clues for gene function, we compared the (mutated) protein sequences of ABCC6/MRP6 with mutations and sequences found in other members of the ABC protein family. We compared sequences and mutations in ABCC6/MRP6 with ABCC2, ABCC8, ABCC7, and ABCR, which are implicated in, respectively, Dubin–Johnson syndrome, familial persistent hyperinsulinaemic hypoglycaemia of infancy, cystic fibrosis, and Stargardt disease or perhaps even age-related macula degeneration.[23–27] As we expected, a number of similar mutations occurred in conserved regions of the ABC proteins or even in the same conserved residues. In all proteins, a large number of mutations implicated in disease occurred in the NBF1 and NBF2 domains. Further alignment showed that the R765Q mutation in ABCC6/MRP6 is the positional equivalent of both the R560T mutation in ABCC7,[28] and the R842G mutation in ABCC8.[29] Similarly, additional possible positional equivalent clusters of conserved and mutated residues were found between ABCC6/MRP6 and ABCC2 (R1114H and R1150H),[30] ABCC6/MRP6 and ABCC7 (3775 del T and W1204X),[31] ABCC6/MRP6 and ABCR (R1459C and H2128R, 4220InsAGAA and R2077W, R1141X and L1631P).[32,33]

Interestingly, for both ABCC7 and ABCR, models were postulated in which the severity of the disease shows an inverse correlation with the predicted transport activity of the ABC protein. According to these models, the mutation type ('mild' or 'severe') of mutations determines the transport activity of ABCC7 and ABCR[34,35] and, consequently, the phenotype. On the basis of our data, we suggest that such a model is not directly applicable to ABCC6/MRP6 and PXE. We observe considerable clinical variability between PXE family members, who, obviously, carry the same mutation, and we suggest that the severity of the PXE phenotype is not directly correlated with the level of ABCC6/MRP6 activity.

Molecular pathology of PXE

The majority of mutations found in ABCC6/MRP6 were nonsense mutations, deletions, as well as missense mutations in conserved regions of the gene. These mutations are expected to result in either a shorter or dysfunctional mRNA or an absent or dysfunctional protein. The latter is most likely compatible with a complete loss of functional ABCC6/MRP6 protein in homozygote patients, and an ar inheritance pattern. Indeed, for three ABCC6/

MRP6 mutations, Ilias *et al*[18] recently found that loss of ABCC6/MRP6 transport activity was directly responsible for PXE.

As confirmed by our data and segregation studies, autosomal recessive segregation was observed in the majority of our PXE families, and may also explain a large part of the so-called sporadic cases. However, there are a number of reports in the literature of families in which PXE potentially segregates in an ad fashion.[6,7] These segregation patterns may result from pseudodominant inheritance, as a result of parental consanguinity, or (mild) manifestation of the disease in heterozygotes.[36–39] On the other hand, the presence of rare ABCC6/MRP6 mutations resulting in a true ad disease and inheritance pattern cannot be ruled out completely. In this study, we presented a novel family with an R1459C ABCC6/MRP6 mutation, in which ad segregation of PXE on the basis of clinical, molecular, and genealogical data, is the most likely explanation for our results.

Further studies using functional (transport) assays in patient fibroblasts, cell lines, or using transgenic mice technology are essential to elucidate the true consequences of the latter and other mutations. Extensive and careful clinical and molecular examination of additional individual PXE patients and pedigrees carrying unique mutations, will, no doubt, contribute to a more complete understanding of the molecular pathology of PXE.

Materials and methods

Clinical examination and human materials

We obtained permission from the Medical Ethical Committee of the Academic Medical Centre in Amsterdam for all studies with human subjects or human material. PXE patients were either ascertained through the national register of genetic eye disease at the Netherlands Ophthalmic Research Institute or through collaboration with physicians. All patients are of Dutch descent.

The diagnosis of PXE in individuals was based on the results of ophthalmological and dermatological examinations. In most patients, histopathological study was performed by skin biopsy, usually from affected skin in the neck, including a von Kossa staining.

Ophthalmological examination included visual acuity measurement, slit-lamp examination, ophthalmoscopy, and frequently fluorescein angiography. Cardiovascular examinations, if performed, included electrocardiograms (ECG). The diagnosis of PXE was considered if there were yellowish papules or plaques on the lateral side of the neck and/or flexural areas of the body, typical histopathological changes in skin biopsy and at least one of the following retinal abnormalities: peaud' orange, angioid streaks, or comets (white punched-out lesions).

Molecular analysis

DNA was isolated from peripheral blood samples of PXE patients and their families according to standard protocols, essentially described elsewhere.[40] Molecular analysis of the ABCC6 gene and FISH analysis were essentially carried out as described.[10]

PCR primers were selected from the published sequence of human chromosome 16 BAC clone A-962B4 (GenBank Accession No. U91318), TIGR database (http://www.tigr.org), or the primers were a gift of collaborators (C Boyd). To distinguish between MRP6 gene and pseudogene sequences, novel primers for exons 1–9 were used, as described elsewhere.[20]

To screen both exon and the adjacent intron sequences, PCR products were derived from intronic sequences 20–50 bp out from the end of each ABCC6/MRP6 exon. PCR was performed on DNA of each PXE patient and the products were analysed with SSCP. Fragments with a mobility shift were characterized by direct sequencing. Heterozygote detection on the ABI-310 was always checked manually by traditional Sanger dideoxy sequencing.

The potential presence of intragenic large deletions of genomic DNA was confirmed by consistent lack of amplification of the relevant exons in patients who were heterozygous or homozygous for deletions. Deletions of exons 15 and 23–29 were detected using primers and methods essentially described elsewhere.[22]

To identify additional putative mutations in patients where we could only identify one or no mutations, Southern blot analysis with ABCC6/MRP6 exons as a probe was carried out, essentially as described.[10] The probes were hybridized against the corresponding genomic DNA of patients that was cut with at least two different restriction enzymes. In addition, we sequenced the promoter region (1 kb before the first ATG) in 14 patients and, finally, we sequenced the entire gene in nine patients. Haplotype analysis with microsatellite DNA markers was carried out in families as described previously.[10] In addition, several new intragenic ABCC6 markers were used (Table 4).

Table 4 Polymorphic sequence changes identified in ABCC6

Nucleotide	*Amino acid*	*Location*	*Estimated frequency (%)*
CA(18)	—	Intron4	68
V415V	1245G>A	10	33
V614A	1841T>C	14	52
T630T	1890C>G	15	22
H632Q	1896C>A	15	24
A830G	2490C>G	19	25
P945P	2846C>T	22	50
L968L	2904G>A	22	20
Int(22)	—	Intron22	50
R1268Q	3808G>A	27	38

The definition of disease-associated alleles essentially follows the criteria described by Le Saux *et al.*[22] In summary, sequence variants predicted to result in nonsense or splice-site changes were considered to be disease-associated alleles if they are absent in DNA of a panel of at least 100 controls. Other variants, such as missense mutations, were considered to be disease associated if they were absent in the same control panel, segregated with the disease in pedigrees, and involve evolutionary conserved amino-acid residues.

Expression studies

Total RNA was isolated from blood and cultured skin fibroblasts from different individuals carrying different ABCC6/MRP6 mutations by use of RNAzol reagent (Biotech. Laboratory). First-strand cDNA was synthesized with Superscript reverse transcriptase (Life Technologies) and oligo T18 primers (Boehringer). cDNA aliquots were subjected to amplification with appropriate primers followed by direct sequencing.

The ABCC6/MRP6-specific RT-PCR primers used to analyse the mutations indicated were as follows: (R1141X): ABCC6/MRP6F, 5′-CTGTCTCCAAGCCATTGGGC-3′ (cDNA position 3008–3027) and ABCC6/MRP6R, 5′-AGCCACCAGTCGCGGGAAAC-3′ (cDNA position 3524–3505); (deletion exon 23–29): ABCC6/MRP6F3, 5′-ATACGGCAGGGTGAAGGCCA-3′ (cDNA position 2801–2820) and ABCC6/MRP6R3, 5′-CAGTGCACTGTGCAAAC CAGC-3′ (cDNA position 4380–4360); (R1459C): ABCC6/MRP6F4, 5′-CTGGCTCTCTGCGGATGAAC-3′ (cDNA position 4081–4100); ABCC6/MRP6R4,5′-AGAACCCGGGCA CAGTCCAT-3′ (cDNA position 4432-4413).

Database sequences and amino-acid sequence alignment

GenBank sequences NP001162 (ABCC6/MRP6), Q92878 (ABCC2), XP004980 (ABCC7) NP00343 (ABCC8), and NP000341 (ABCR) were used to construct an alignment of the ABCC protein sequences and analyse the evolutionary conservation of corresponding amino-acid positions. Sequences were aligned using CLUSTAL algorithm.

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Chapter 3

Analysis of the frequent R1141X mutation in the ABCC6 gene in Pseudoxanthoma Elasticum

Analysis of the Frequent R1141X Mutation in the *ABCC6* Gene in Pseudoxanthoma Elasticum

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Purpose. To characterize the *ABCC6* R1141X nonsense mutation, which is implicated in more than 25% of a cohort of patients from The Netherlands with pseudoxanthoma elasticum (PXE).

Methods. A combination of single-strand conformational polymorphism (SSCP), PCR, sequencing, and Southern blot analysis was used to identify mutations in the *ABCC6* gene in 62 patients. Haplotypes of 16 patients with the R1141X mutation were determined with eight polymorphic markers spanning the *ABCC6* locus. The effect of the R1141X mutation on the expression of *ABCC6* was studied in leukocytes and cultured dermal fibroblasts from affected skin in patients heterozygous or homozygous for the R1141X mutation. *ABCC6* expression was analyzed by RT-PCR and immunocytochemistry with *ABCC6*-specific monoclonal antibodies.

Results. The *ABCC6* R1141X mutation was found on 19 alleles in 16 patients with PXE and occurred in heterozygous, homozygous, or compound heterozygous form. All R1141X alleles were associated with a common haplotype, covering at least three intragenic *ABCC6* markers. None of the patients or healthy control subjects had a similar *ABCC6* haplotype. Furthermore, the results showed that the expression of the normal allele in R1141X heterozygotes was predominant, whereas no detectable, or very low, *ABCC6* mRNA levels were found in R1141X homozygotes. Immunocytochemical staining of cultured dermal fibroblasts with *ABCC6*-specific monoclonal antibodies showed no evidence of the presence of a truncated protein in patients with PXE who were homozygous for R1141X.

Conclusions. A specific founder effect for the R1141X mutation exists in Dutch patients with PXE. The R1141X mutation induces instability of the aberrant mRNA. Functional haploinsufficiency or loss of function of *ABCC6* caused by mechanisms, such as nonsense-mediated decay (NMD), may be involved in the PXE phenotype. (*Invest Ophthalmol Vis Sci.* 2003;44:1824–1829) DOI:10.1167/iovs.02-0981

Pseudoxanthoma elasticum (PXE) is an autosomal inherited disorder. Patients have a spectrum of ocular abnormalities, skin lesions, and various cardiovascular complications.

Ocular signs eventually develop in most patients with PXE. Initially, eye abnormalities consist of peau d'orange or mottled hyperpigmentation of the retina. Subsequently, cometlike streaks, pinpoint white lesions of the choroid, sometimes with a hypopigmented tail in the retinal pigment epithelium, also called comets, and angioid streaks appear. Angioid streaks are cracks in the aging Bruch's membrane that most often radiate from the disc in a manner mimicking blood vessels and frequently a site of subretinal neovascular growth from the choroid. Eventually, a disciform macular degeneration develops that is frequently devastating to central vision.

Eye, skin, and cardiovascular abnormalities all appear to result from mineralization and calcification of elastic fibers in connective tissue of the affected tissues and organs, including the internal elastic lamina of Bruch's membrane.[1,2]

We and others localized the PXE gene to chromosome 16, location p13.1[3–5] and implicated *ABCC6* gene mutations in all genetic forms of PXE.[6–8] The *ABCC6* gene, formerly called multidrug resistance protein 6 (*MRP6*), is a member of the adenosine triphosphate (ATP)–binding cassette (ABC) transporter superfamily C and encodes a 1503-amino-acid transmembrane protein.[9] The protein is composed of three hydrophobic membrane-spanning domains, 17 transmembrane spanning helices and two evolutionary conserved nucleotide binding folds (NBF1 and NBF2).[10] In humans, *ABCC6* is mainly expressed in liver and kidney.[6,11,12] Using semiquantitative RT-PCR, we have found low *ABCC6* expression levels in the retina, as well as in other tissues usually affected by PXE.[6] Recently, we have developed several monoclonal antibodies against *ABCC6* and localized the protein to the basolateral side of human hepatocytes and renal epithelial cells.[12] Evidence was obtained that *ABCC6* transports glutathione conjugates, including leukotrien-C4 (LTC4) and *N*-ethylmaleimide *S*-glutathione (NEM-GS).[13] Loss of *ABCC6* function associated with three mutations was found to be involved in PXE.[13] However, the functional relationship between specific substrates transported by *ABCC6* and the accumulation of abnormal elastic fibers in PXE remains to be elucidated.

At least 57 distinct *ABCC6* mutations associated with PXE have been observed in different populations. These include nonsense, missense, and putative splice site mutations, as well as deletions and an insertion.[6,7,8,14,15] Most mutations were located toward the carboxy terminal end of the protein, and formed three clusters: in the NBF1 domain, in the 8th cytoplasmic loop between the 15th and 16th transmembrane regions, and in NBF2. The frequency of mutations in the eighth cytoplasmic loop was higher than those in the NBF1 and NBF2 domains,[15] which suggests that this domain is critical for normal protein function. Although the autosomal recessive form of PXE (arPXE) is the predominant form of the disease, differ-

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ent molecular mechanisms may be involved in various types of PXE. No clear phenotype-genotype correlation has been established for the *ABCC6* gene mutations so far. The mechanism of calcification of elastic fibers in Bruch's membrane and skin is not known. Therefore, it is important to study specific *ABCC6* gene mutations in more detail at both the RNA and protein levels. Such studies may contribute to the understanding of the pathologic molecular cause underlying PXE and the clarifying of the functionally important regions of the protein.

In this study, the recurrence of the R1141X mutation in 16 patients with PXE and the level of expression of *ABCC6* mRNA with the R1141X mutation in (PXE) leukocytes and (PXE) fibroblasts were analyzed. In addition, the ABCC6 protein in cultured dermal fibroblasts from a patient homozygous for R1141X was studied with *ABCC6*-specific monoclonal antibodies.

Materials and Methods

Patients

PXE-affected families and sporadic cases were recruited with informed consent from the Medical Ethical Committee of the Academic Medical Center in Amsterdam. They were of Dutch descent and were primarily ascertained through the national register of genetic eye diseases at The Netherlands Ophthalmic Research Institute. Sixteen Dutch families and patients with the R1141X mutation were included in the study. Genealogical studies show that patients were not related up to four prior generations.

The diagnosis of PXE in individuals was based on the results of dermatological and ophthalmologic examinations. Most patients had obvious skin lesions. In six patients without any obvious skin lesions, skin biopsy specimens were taken from the neck, and sections were stained by the von Kossa method. In 15 patients, recent ophthalmologic examination included visual acuity measurement, slit lamp examination, ophthalmoscopy, and often fluorescein angiography. In one patient, medical records indicated the presence of angioid streaks. In six patients, cardiovascular examinations were performed that included electrocardiograms (ECGs). Control subjects for mutational analyses were spouses of (PXE and other) patients of The Netherlands Ophthalmic Research Institute and the Academic Medical Center in Amsterdam. Definition of ethnic origin of each subject was based on the country of birth in four generations. All investigations adhered to the tenets set forth by the Declaration of Helsinki.

Mutation Analysis

Genomic DNAs were prepared from patients' peripheral blood lymphocytes according to standard procedures. Primers used for polymerase chain reactions were selected from the published sequence of the human chromosome 16 BAC clone A-962B4 (GenBank Accession No. U91318; http://www.ncbi.nlm.nih.gov/Genbank; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD) and the TIGR database (http://www.tigr.org; provided in the public domain by The Institute for Genomic Research [TIGR], Rockville, MD). Fragments with a mobility shift in a single-strand conformational polymorphism (SSCP) assay were sequenced. The PCR, SSCP analysis, and cycle sequencing were essentially performed as we described previously.[6] Potential intragenic deletions of genomic DNA were confirmed either by Southern blot analysis, familial segregation of CA repeats, or the consistent lack of amplification of the relevant exons. All patients with one mutant allele were analyzed further by hybridization on *Eco*RI-digested and *Pst*I-digested genomic DNA using the amplified *ABCC6* exons from genomic DNA as probes. In addition, the *ABCC6* promoter region was sequenced up to 1 kb upstream of the first ATG. All coding sequences were analyzed in more than 100 healthy individuals, to distinguish between disease-causing mutations and polymorphic variants.

Restriction Analysis of the R1141X Mutation and Polymorphisms

After identification of the R1141X mutation and detection of other intragenic polymorphisms, optimized protocols were designed by using PCR and restriction analyses. The R1141X mutation leads to the loss of a *Bst*YI restriction site, which was confirmed by a restriction fragment length polymorphism assay. The *Bst*YI enzyme cleaves the wild-type sequence but not the mutant sequence. Similarly, the intragenic polymorphisms, 1896 C→A, 2490 C→G, and 3803 G→A were determined by the digestion of PCR fragments with enzyme *Hpy*CH4III, *Hea*III, and *Bst*NI, respectively. The digested products were separated on 3% agarose gels. Allele frequencies for identified mutations and polymorphisms were determined both in our cohort of patients with PXE and in more than 100 control chromosomes from our healthy study participants.

Haplotype Analysis and Assessment of Founder Effect

We constructed haplotypes with eight polymorphic markers, which span ~1.5 cM around the *ABCC6* gene on chromosome 16 at location p13.1. These included the *ABCC6* flanking markers *D16S3060*, *972AAAG1*, *962CA2*, and *D16S764* as well as the intragenic markers 3803 G→A, 2490 C→G, 1896 C→A, and CA(18). Markers were analyzed on 6% polyacrylamide gels or on 3% agarose gels after enzyme digestion. We constructed (phase-known) haplotypes of all informative alleles from patients with the R1141X mutation. Control haplotypes were determined on the basis of the allele segregation within individual families. Assessments of founder haplotypes were based on χ^2 analyses.[16]

Fibroblast Cultures

For functional studies, primary fibroblast cultures were established from dermal biopsy specimens of affected skin of patients with PXE and healthy control subjects. Cells were grown in RPMI (Life Technologies, Gaithersburg, MD), containing 10% fetal calf serum, and were used between the second and fourth passages. Total RNA was isolated from subconfluent cultures.

RT-PCR Analysis of the *ABCC6* Transcript

Total RNA was prepared from cultured dermal fibroblasts and peripheral blood leukocytes of patients with PXE and healthy control subjects, using the reagent (RNAzol; Cinna/Biotech Laboratories, Houston, TX). For RT-PCR analysis, 4 μg of total RNA was reverse-transcribed in the presence of oligo$(dT)_{12\text{-}18}$ and 200 U reverse transcriptase (Superscript II RT; Life Technologies). Part of the RT reaction product was used as a template in a PCR reaction with *ABCC6* cDNA-specific primers spanning exon 24.[6] RT-PCR of β-actin[6] served as a control. In heterozygotes, the ratio of expression between the wild-type and the mutant *ABCC6* allele in fibroblasts or in leukocytes was determined by cloning a mutation-specific RT-PCR product into a vector (pGEMTeasy; Promega, Madison, WI) followed by transformation of *Escherichia coli* strain DH5α. The presence or absence of the R1141X mutation in individual clones was checked with digestion with *Bst*YI, and, accordingly, the inserts of individual clones were assigned to be expression products from the mutant or wild-type allele.

Immunocytochemistry

Dermal fibroblasts were seeded onto sterile glass coverslips and cultured for an additional 24 hours. Cells were washed with PBS, fixed for 7 minutes in acetone at room temperature, incubated with primary rat monoclonal antibodies (1:10) for 1 hour (in the presence of 2% normal rabbit serum), washed, and further incubated with a Cy3-labeled goat anti-rat secondary antibody (1:400, Jackson ImmunoResearch, West Grove, PA). The slides were examined under a fluorescence microscope (Leica DMRB, Heidelberg, Germany). The monoclonal antibod-

TABLE 1. Genotype and Clinical Features of 16 Patients with the R1141X Mutation

Pedigree	Allele 1	Allele 2	Skin	Eyes	Cardiovascular
25494	R1141X	Delex23–29	+	AS, MD	HT
26026	R1141X	Delex23–29	+, b+	D	D
26241	R1141X	Delex23–29	+, b+	PdO, AS	N
26007	R1141X	1944del22	+	RPE changes	N
26240	R1141X	Q749X	+, b+	AS	MVP
26273	R1141X	3775delT	D	AS, neo	D
26091	R1141X	R1141X	+, b+	AS	GI hemorrhage
26101	R1141X	R1141X	+	AS	TVI
26107	R1141X	R1141X	D	Neo	D
26093	R1141X	WT	+	AS	N
26123	R1141X	WT	+, b+	PdO, comet	N
24694	R1141X	WT	+	AS, MD	CI
25908	R1141X	WT	+	AS	D
26109	R1141X	WT	+	AS, neo	D
26215	R1141X	WT	+	+	D
26242	R1141X	WT	+, b+	PdO	MVP

WT, wild type; +, affected; b+, biopsy specimen obtained and histologic PXE changes determined after von Kossa staining; D, declined and/or no data available; AS, angioid streaks, MD, macula degeneration, PdO, peau d'orange, RPE involvement; neo, neovascularisation; comet, presence of comets; HT, hypertension; N, normal, MVP, mitral valve prolaps; GI, gastrointestinal; TVI, tricuspid valve insufficiency; CI, cerebral infarct.

ies for immunocytochemical staining were M_6II-7, MRPr1, and M5I-1 reactive to ABCC6, ABCC1, and ABCC5, respectively.[11,12,17]

RESULTS

R1141X Mutation Analysis

Mutation analysis of the *ABCC6* gene resulted in the identification of the R1141X mutation, which accounted for up to 30% of all mutations detected in the *ABCC6* gene in our cohort of patients with PXE. The R1141X mutation was caused by a C→T substitution at nucleotide 3421 in the putative eighth intracellular loop. This mutation produces a stop codon at 1141 instead of an arginine residue and results in a shorter mRNA and, theoretically, in a C-terminal–truncated protein that lacks part of the transmembrane domain and the second ATP-binding domain. Previously, we found that the R1141X mutation was associated with a strong increase in the prevalence of coronary artery disease.[18]

In our PXE cohort, R1141X was found in 19 of 29 nondeletion alleles in DNA of 16 patients with PXE. A summary of the results is presented in Table 1. In three patients, the R1141X mutation was observed in a homozygous form; in seven, it was in heterozygous form; and in six, it occurred in combination with other mutations in the second allele in compound heterozygous form.

In three of these six compound heterozygotes (from family pedigree [P]25494, P26026, P26241), an additional intragenic 16.5-kb deletion was present. When we sequenced the fragments spanning the deletion region, we found the same break points in all three patients at nucleotide 47322 within intron 22 and nucleotide 30869 within intron 29. This seven-exon deletion is predicted to result in a polypeptide that lacks 405 amino acids.

In the three other compound heterozygotes, the combination R1141X with a del22 bp in exon 16, a 3375delT, or Q749X was identified. In family P26007, the proband showed a 22-bp deletion in exon 16, which created a premature stop codon at position 688, and which resulted in the loss of eight amino acids (Arg-Ile-Asn-Leu-Thr-Val-Pro-Glu [648-655]) of putative protein sequence. In family P26273, the proband inherited a maternal deletion of a T at cDNA position 3775 in exon 27, which resulted in a frameshift at codon 1259 and premature termination at codon 1272. The last patient, P26240, inherited the R1141X mutation and another nonsense mutation, Q749X. The latter mutation occurred in NBF1 of the ABCC6 protein.

Founder Effect for the R1141X Mutation

To determine whether the R1141X mutation originates from recurrent de novo mutational events or from founder effects, *ABCC6*-associated haplotypes of all patients carrying the R1141X mutation and of control subjects were constructed. Characteristics and frequency of eight markers, which spanned approximately 1.5 cM of DNA, are summarized in Table 2.

We detected 11 polymorphisms in the exonic *ABCC6* sequences. Three of these were used in this study to construct the following haplotypes: (1) 1896 C→A in exon 15 predicting an H632Q substitution, (2) 2490 C→G in exon 19 predicting an A830G substitution, and (3) 3803 G→A in exon 27 predicting a R1268Q substitution. These dimorphisms could easily be detected by PCR and restriction analysis (Fig. 1). The C→A change at nucleotide 1896 in exon 15 occurred in 24 of 32 alleles (75.0%) in the patients and 80 of 204 alleles (39.2%) in the control subjects. Statistical analysis showed a significant difference between the patients and the control subjects (χ^2 = 32.1, $P < 0.001$). The second dimorphism (2490 C→G) occurred frequently in the patient group (86.2%) but rarely in the control group (14.2%). Highly significant differences were found between patients and control subjects for this dimorphism (χ^2 = 135.3, $P < 0.0001$). The dimorphism (3803 G→A) revealed no differences between the patient and control groups. Both groups had 30% wild-type alleles.

The minimum common haplotype shared by all 19 alleles with the R1141X mutation was represented by the haplotype G(3803G→A)-G(2490C→G)-A(1896C→A) (Table 2). None of 10 control subjects carried this haplotype. In 17 patients, this common haplotype (G-G-A) was extended to at least one extra marker allele, allele 4 (962CA2), distal to *ABCC6*. Thirteen of the latter group shared at least one intragenic marker allele, CA(18), located close to the 5′ end of the *ABCC6* gene, or even larger regions around the disease-related gene.

TABLE 2. Haplotype of the R1141X Mutation Alleles

	Marker								
Pedigree	D16S 3060	972AA AG1	962 CA2	*3803 G→A*	**3421* C→T*	*2490 C→G*	*1896 C→A*	*CA (18)*	D16S 764
25908	7	3	4	G	T	G	A	1	3
26273	5	3	4	G	T	G	A	1	3
25494	2	3	4	G	T	G	A	1	3
26240	3	2	4	G	T	G	A	1	3
24694	1	2	4	G	T	G	A	1	3
26109		2	4	G	T	G	A	1	3
26241	4	2	4	G	T	G	A	1	3
26101	2	3	4	G	T	G	A	1	2
26101	5	3	4	G	T	G	A	5	1
26026	4	3	4	G	T	G	A	1	1
26091	4	3	4	G	T	G	A	1	1
26091	4	12	4	G	T	G	A	1	2
26093	2	2	4	G	T	G	A	1	1
26007	4	12	4	G	T	G	A	1	2
26107	2	13	4	G	T	G	A	3	2
26107	2	13	4	G	T	G	A	3	3
26123	2	2	4	G	T	G	A	5	4
26215	1	2	3	G	T	G	A	4	3
26242	2	3	2	G	T	G	A	4	2

Distinct alleles are indicated by number. Identical haplotypes for the R1141X mutation are shaded. Marker alleles present within the *ABCC6* gene are presented in italic. Underline shows homozygosity for the R1141X mutation in the proband(s).

*R1141X mutation.

Effect of R1141X on *ABCC6* Expression in Dermal Fibroblasts

The *ABCC6* mRNA with the R1141X mutation encodes a C-terminally truncated ABCC6 protein that lacks part of one of the transmembrane domains and one of the ATP-binding cassette domains. To determine the effect of this mutation on the expression of the *ABCC6* gene, we analyzed dermal fibroblasts from individuals homozygous or heterozygous for R1141X and healthy control subjects. The presence of the mutations was confirmed by PCR amplification of exon 24 containing the R1141X mutation followed by restriction fragment length polymorphism analysis (Fig. 2) and direct sequencing (not shown). Next, we analyzed the amount of mRNA from alleles with the R1141X mutation. RT-PCR analyses of cultured PXE fibroblasts with the R1141X in homozygous form did not contain detectable *ABCC6* mRNA. Fibroblasts from those heterozygous for the R1141X mutation appeared to have a reduced level of *ABCC6* mRNA compared with the healthy control subjects (Fig. 3). The latter result indicates that the R1141X mutation affects the abundance of the mutant mRNA. To examine this in more detail, we determined the ratio of steady state transcript levels between wild-type and mutated mRNA in mononuclear blood cells and fibroblasts of patients heterozygous for the R1141X mutation. As a control for the latter analysis, we also determined this ratio in blood cells from a healthy donor and from a patient with PXE heterozygous for a missense mutation R1459C in exon 30 of *ABCC6*. In fibroblasts of patients heterozygous for the R1141X mutation, no mRNA containing the mutation was found, whereas in blood cells, only 5% of the ABCC6 mRNA was from the mutated allele (Table 3). In contrast, the relative amount of mRNA carrying the R1459C mutation (48%) was very similar to that of the wild-type mRNA (52%; Table 3), whereas the cells of a healthy donor contained only wild-type mRNA.

In parallel experiments, we determined the expression of the R1141X truncated protein in cells of patients with PXE. Cultured dermal fibroblasts of a patient with PXE homozygous for the R1141X mutation and that of a healthy control subject were analyzed by immunocytochemistry. As is shown in Figure 4, staining with a monoclonal antibody against *ABCC6* showed only staining of the control fibroblasts but not of the PXE fibroblasts. Incubation with a monoclonal antibody against *ABCC1* indicated that this protein was expressed in both normal and PXE fibroblasts at similar levels. *ABCC5* appeared not to be expressed at detectable levels by human fibroblasts, as

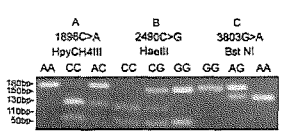

FIGURE 1. Allele-specific restriction analysis of the three dimorphisms in the *ABCC6* gene. PCR fragments were amplified using primers on exon 15, 19, and 27, followed by digestion with *Hpy*CH4III, *Hae*III, or *Bst*NI, respectively. (**A**) DNA fragments of exon 15 were digested by *Hpy*CH4III, which cuts the sequence only if a C is present at position 1896C in the sequence. (**B**) DNA fragments of exon 19 were digested by *Hae*III, which cuts the sequence only if a C is present at cDNA position 2490. (**C**) PCR fragments of exon 27 were digested with *Bst*NI, which cuts the sequence only if an A is present at position 3803. The dimorphisms and the restriction enzymes by which wild-type and variation allele were separated are depicted. The possible genotypes for each polymorphism are illustrated.

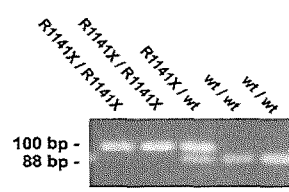

FIGURE 2. The presence of the R1141X mutation was confirmed by digestion with *Bsf*YI of PCR products containing exon 24. Digestion products were size fractionated on 3% agarose gel. For the wild-type allele, this results in an 88- and a 12-bp fragment. The R1141X mutant allele leads to the loss of the *Bsf*YI restriction site and produces a single fragment of 100 bp. The 12-bp fragment is not visible.

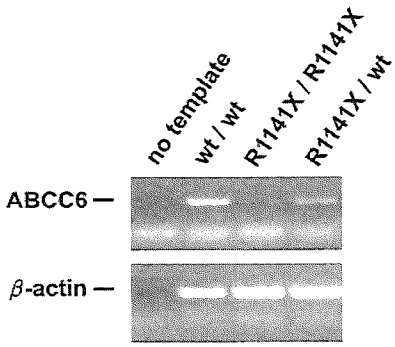

FIGURE 3. *Top*: RT-PCR analysis of *ABCC6* expression in cultured dermal fibroblasts from patients heterozygous or homozygous for the R1141X mutation. Fibroblasts from a healthy donor were also analyzed. *Bottom*: shows the control PCR for β-actin expression.

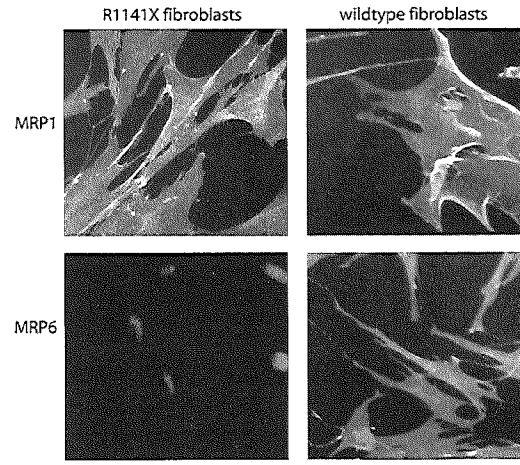

FIGURE 4. Staining with MRP1 and MRP6 monoclonal antibodies of a monolayer of dermal fibroblasts from a healthy individual and a patient with PXE heterozygous for the R1141X mutation.

incubation with a monoclonal antibody against this protein gave no staining above background (not shown). These results suggest that the R1141X mutation in *ABCC6* does not lead to detectable amounts of truncated protein.

Phenotypes of Patients with the R1141X Mutation

A summary of clinical data available from the 16 patients with PXE with R1141X mutations is shown in Table 1. All probands had either clinically obvious PXE skin lesions or had typical PXE abnormalities detected by von Kossa staining. Angioid streaks were present in 10 patients. Six patients from the latter group had neovascularization or macular degeneration. Variable cardiovascular abnormalities were detected in six patients.

In summary, in all patients homozygous or compound heterozygous for the R1141X mutation, we observed ocular and skin abnormalities and, less frequently, cardiovascular problems. However, because the expression of the disease in these tissues is highly variable among our patients, we could not correlate a distinct phenotype with the R1141X mutation.

DISCUSSION

R1141X Mutation Analysis

We detected the R1141X mutation in homozygous, heterozygous, and compound heterozygous forms. In nine patients the R1141X mutation was present in a homozygous form or a compound heterozygous form. This is compatible with the frequently observed autosomal recessive inheritance of the disease. In seven patients, we detected R1141X in heterozygous form. These patients were either sporadic or were members of families in which autosomal recessive inheritance was the most likely segregation pattern. However, despite extensive screening, we have not yet found another mutation or deletion in the second, non-R1141X, *ABCC6* allele. Given a mutation detection frequency of approximately 50% to 55.3% (mutations per allele) in European cohorts, the most likely explanation of our results is that we still missed a substantial amount of mutations. Consequently, the pathologic molecular aspect of the R1141X mutation is most likely compatible with the frequently occurring autosomal recessive inheritance in PXE. Nonetheless, potential mild expression of the disease in carriers of the R1141X mutation warrants further investigation.

TABLE 3. The Expression Ratio of *ABCC6* Wild-Type and Mutated mRNA

Genotype	Tissue	WT Allele (%)	Mutant Allele (%)
WT/WT	Blood	20/20 (100)	No
R1141X/WT	Blood	38/40 (95)	2/40 (5)
R1459C/WT	Blood	52/100 (52)	48/100 (48)

The number of PXE heterozygotes carrying an *ABCC6* R1141X (R1141/X) or R1459C (R1459C/WT) mutation and a healthy control subject with wild-type *ABCC6* (WT/WT).

Data are number of subjects/total in group, with the percentage of the total group in parentheses.

Founder Effect for the R1141X Mutation

Mutation analysis of the *ABCC6* gene in patients with PXE has yielded 57 different *ABCC6* mutations to date.[15] The R1141X mutation was reported to be the most common mutation by us and others, especially in European patients.[14,15] Recently, we also found that R1141X may be associated with a strong increase in the prevalence of coronary artery disease.[18] The association between its high frequency and the geographical distribution could reflect a founder effect from a common ancestor. To test this hypothesis, we analyzed the R1141X mutation in more detail in this study.

The majority of our R1141X mutant alleles (17/19) shared a common haplotype spanning at least one *ABCC6* flanking marker. Our results and statistical analysis suggested that a founder effect exists in the Dutch PXE group. In only two patients did partial aberrations of the consensus haplotype occur. These could be due to (ancient) recombination events including CA(18), 972AAAG1, and D16S764.

Identification of founder effects in the local population, as presented in this study, can greatly simplify genetic analysis of the disease, because, initially, the founder mutation can be rapidly screened in all patients. Associated clinical studies may provide further accurate information for genetic counseling and prenatal diagnosis.

Predominant Expression of the Normal *ABCC6* Allele in Patients with PXE Heterozygous for the R1141X Mutation

For several mammalian mRNAs, it has been shown that a nonsense mutation or a frameshift mutation that generates a nonsense codon may greatly influence the abundance of these transcripts. A specific mechanism called nonsense-mediated mRNA decay (NMD) accelerates decay of transcripts coding for truncated proteins and thus minimizes potential metabolic damage.[19,20] We found no detectable *ABCC6* mRNA in patients with PXE who were homozygous for the R1141X mutation. Consistent with this observation, no ABCC6 protein was detected in cultured dermal fibroblasts of a patient homozygous for R1141X. Using a more quantitative approach, we found that in cultured dermal fibroblasts of a R1141X heterozygote, only transcripts from the wild-type allele were detected. In mononuclear blood cells of a R1141X heterozygote the mutated transcript was detected, but the abundance was reduced to 5% of total ABCC6 mRNA.

Our results suggest that the R1141X mutation induces instability of the aberrant *ABCC6* mRNA, which leads to a reduced abundance of the corresponding transcript due to alterations in RNA processing by NMD. The latter mechanism may in part be an explanation of the obvious variability in the expression of the disease. The possibility that NMD contributes to a particular phenotype has also been suggested for other genes, such as fibrillin 1 in Marfan syndrome[21] and β-globin in beta zero-thalassemia.[22,23] So far, because of the small size of the families, we have not established a clear correlation between the level of *ABCC6* mRNA and the patient's phenotype. However, it is reasonable to assume that dosage-dependent severity caused by the presence of NMD of mRNA may be involved in PXE. Complete loss of *ABCC6* function causes PXE in homozygotes or compound heterozygotes, whereas partial loss of *ABCC6* function in heterozygotes may result in a variable phenotype ranging from no signs at all to the complete PXE phenotype.

Features of the Phenotype

The clinical variability in PXE was demonstrated previously[2,24] and also occurred in our R1141X patient cohort. In this study, we could not firmly predict the phenotype from the genotype, or vice versa. The correlation between genotype and phenotype may be obscured by several factors. The small size of our cohort limited the evaluation of the genotype and phenotype correlation. In addition, additional unknown environmental, metabolic, or genetic determinants may modify the phenotype. In future studies, we have to investigate the PXE phenotype in a thorough prospective way to obtain any significant clues for genotype-phenotype relationships.

Conclusion

In summary, this study presents evidence that the frequent occurrence of the *ABCC6* R1141X mutation in Dutch patients with PXE was due to a founder effect. The PXE phenotype of the R1141X mutation is most likely due to a complete loss of function or functional haploinsufficiency of the *ABCC6* gene. No clear correlation between the R1141X genotype and phenotype could be established in the cohort studied. Further analysis of additional PXE families with this mutation should help to increase our understanding of the function of the *ABCC6* gene and the molecular pathology underlying PXE.

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Chapter 4

Efficient Molecular Diagnostic Strategy for ABCC6 in Pseudoxanthoma Elasticum

Abstract

Pseudoxanthoma elasticum is a hereditary disorder of the connective tissue with skin, cardiovascular, and visual involvement. In familial cases, PXE usually segregates in an autosomal recessive (AR) fashion. The aim of this manuscript is to describe an efficient strategy for DNA diagnosis of PXE.

The two most frequent mutations, R1141X and an ABCC6 del exons 23-29, as well as a core set of mutations were identified by restriction enzyme digestion and size separation on agarose gels. Next, in the remaining patient group in which only one or no mutant allele was found, the complete coding sequence was analysed using the denaturing high performance liquid chromatography (DHPLC). All variations found were confirmed by direct DNA sequencing. Finally, Southern blot was used to investigate the potential presence of small or large deletions. 20 different mutations including two novel mutations in the *ABCC6* gene were identified in 80.3% of the 76 patients, and 58.6% of the 152 *ABCC6* alleles analysed. With this strategy, 70 (78.7%) out of 89 mutant alleles could be detected within a week. Conclusion: This strategy leads to both reliable and time-saving screening for mutations in the *ABCC6* gene in sporadic cases and in families with PXE.

Introduction

Pseudoxanthoma elasticum (PXE) is an inherited connective tissue disorder marked by progressive calcification and mineralization of elastic fibres of the skin, retina, and the cardiovascular system (Goodman et al. 1963; Lebwohl et al. 1993; McKusick 1972). Patients frequently have dermal lesions, experience progressive loss of visual acuity, and are at increased risk for cardiovascular complications (Trip et al. 2002). At present, PXE is incurable and appears to occur in all of the world's populations with an estimated prevalence ranging from 1/25,000 to 1/100,000 (www.pxe.org). Since it was disclosed that mutations in the *ABCC6* gene are responsible for PXE (Bergen et al. 2000; Le Saux et al 2000; Ringpfeil et al. 2000), the diagnosis of PXE depends more and more on molecular diagnosis.

The detection of mutations in the *ABCC6* gene is complicated by the presence of large *ABCC6* associated deletions and the presence of *ABCC6* pseudo genes in

the human genome (Cai et al. 2001; Pulkkinen et al. 2001). *ABCC6* mutations associated with PXE have been identified in 21 out of 31 exons (Le Saux et al. 2001; Hu et al.2003a). The mutations identified range in size from single base pair substitutions to large genomic deletions, involving several exons of *ABCC6* or the entire gene (Bergen et al.2000; Le Saux et al. 2001; Hu et al. 2003a). The challenge for molecular diagnostics is to set up a fast and cheap analytical method with high sensitivity and specificity. A broadly and traditionally used screening method is single strand conformational polymorphism (SSCP). However, SSCP requires more than one set of running conditions to achieve maximum sensitivity and it is limited by fragment size. Screening for all 31 exons in the ABCC6 gene in each new patient sample with SSCP is very time consuming and not efficient to offer it as a routine molecular diagnostic service. Based on our experience with ABCC6 mutations, we described in this study an efficient, cost-effective, and time-saving screening strategy combining different technical approaches for the molecular diagnosis of PXE.

Materials and Methods

Patients

PXE patients (n=76, 59 patients described previously,[7] and 17 new cases) were recruited through the national register of genetic eye disease at the Netherlands Ophthalmic Research Institute (NORI). All patients were of Dutch descent and informed consent was obtained from each one. All patients had ophthalmological examination including visual acuity measurement, slit-lamp examination, ophthalmoscopy, and frequently fluorescein angiography. In most patients, a skin biopsy was taken from affected skin, followed by histopathological study with von Kossa staining. Cardiovascular examinations, if performed, included electrocadiograms (ECG). The diagnosis of PXE was made when skin signs or typical calcification of elastic fibres in skin biopsy were present, together with one of the following retinal abnormalities: peau d' orange, angioid streaks (with or without macular degeneration), or comet-like lesions. Control subjects for mutational analyses were spouses of (PXE and other) patients of The Netherlands Ophthalmic Research Institute and the Academic Medical Centre (AMC) in Amsterdam.

DNA amplification

DNA was isolated from peripheral blood by the salt precipitation method. PCR primers were designed according to the published sequence of human chromosome 16 BAC clone A-962-B4 (GenBank Accession No. U91318), TIGR database (http://www.tigr.org). Primers designed for each exon contained at least a 20bp flanking intronic sequence (Table 1). To distinguish between *ABCC6* gene and pseudogene sequences, primers of exon 1-2 and 5-8 were designed according to Pulkkinen et al. (2001), to exclude the pseudogenes of *ABCC6,* as described. Both functional and pseudo-gene sequences were amplified in fragments of exons 3, 4, and 9. Whenever necessary, sequence analysis was carried out with primers corresponding to the real gene only (Pulkkinen et al. 2001). The deletion in ABCC6 exons 23-29 was detected using following primers: primer 29a, 5'ctgtaggcaggtcattcaaa 3'; primer 29w, 5' gtggatcacctgagagtttc 3'; primer 22m, tcccctaaagatggagagat 3'. These three primers can amplify both wild type DNA and exon 23-29 deletion alleles in single PCR reaction using an annealing temperature of 55 °C. Primers used to detect the deletion of exon 15 were designed and used as described previously (Le Saux et al. 2001).

All primer pairs were amplified by using 30ng DNA in a 50µl reaction containing 5µl 10x PCR buffer, 0.5 mM dNTPs, 50ng each of primers, and 0.5U Taq polymerase (Applied Biosystems). Amplification was started for 5 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at annealing temperature presented in table 1 and 1 min at 72°C, terminating with a 5 min extension at 72°C.

Restriction digestion and gel electrophoresis

Mutation detection protocols were optimized by using multiple rounds of slightly different PCRs and restriction analyses. Six nucleotide changes, including 2294g>a (R765Q in exon 18), 3421c>t (R1141X in exon 24), 3904g>a (G1302R in exon 28), 3907g>c (A1303P in exon 28), 3775del T (W1259 frame shift in exon 27), and 4377c>t (R1459C in exon 30) were determined by the digestion of PCR fragments with restriction enzymes SmaI, BsiYI, NciI, HeaIII, BstNI, and AciI, respectively. The restriction reactions were carried out according to the manufacturer's recommendations (Biolabs). The digested products were separated on 3% agarose gels. Allele frequencies for mutations identified were determined both in patients and at least 100 control chromosomes.

Several mutations including del exons 23-29, del exon 15, 1944del22 in exon 16, and 3821del 48 in exon 27 were detected by amplification of appropriate PCR fragments. Different sized PCR fragments, corresponding to mutated alleles, could be detected by electrophoresis on 2-4% agarose gels containing ethidium bromide. The fragments were visualized by ultraviolet light.

Mutation screening by DHPLC

The amplification efficiency of PCR products from patients and control subjects was checked on agarose gel. The PCR product from each patient was mixed with equal volumes of PCR product from a control person. Mixed samples were heated at 95°C for 5 min, cooled down to 34 °C at –1.5 °C /min to allow heteroduplex formation. 8 to 10µl of each sample was run on a Transgenomics Wave Genetic Analyser. DNA amplification products of sequence variations previously determined by sequencing were used as positive controls. Optimal strand separation temperature was determined for each fragment by Wavemaker ™-software. DHPLC analysis was conducted at two to three temperatures. The optimal temperatures were chosen based on our test runs with amplicons from each exon with known variations. For fragments without positive control, we used at least two of the temperatures recommended by the software. Since the PCR of exons 3, 4, and 9 also amplified sequences of the *ABCC6* pseudogene, heteroduplexes for WAVE mutation detection can be formed between the pseudogene and the functional gene sequences. Consequently, the latter samples were not mixed with control DNA, before they were analysed on DHPLC.

Direct sequencing

Genomic DNA was amplified and products were purified on Microcon 100 columns. Direct sequencing was carried out with the Big Dye Terminator Cycle Sequencing kit from Applied Biosystems. Cycle sequencing was performed in 20µl containing 20ng template, 3 pmol selected primer with the Big Dye terminator Version 2 sequencing kit (PE Applied Biosystems) mix as supplied by the manufacturer. An aliquot of 15µl Template Suppression Reagent (TSR) was added to the vacuum-dried purified reaction product. The products were denatured at 94°C for 2min and kept on ice before fragment detection on an ABI-310 (Applied Biosystems). Heterozygote detection on the ABI-310 was always checked manually by traditional Sanger dideoxy sequencing.

Southern blot

To identify additional putative deletions or duplications in DNA of PXE patients, Southern blot analysis was carried out using one or more of the amplified *ABCC6* exons from genomic DNA as probes. Samples of genomic DNA (5-10μg) were digested with EcoRI and PstI according to the manufacturer's recommendations (Biolabs). Digests were electrophoresed in agarose gels and blotted on GeneScreen Plus nylon membranes (NEN). Of a human ABCC6/MRP6 genomic DNA probe 50 ng was labelled with 32^{P}-dCTP by multiprime labelling (Amersham labelling system). Hybridizations were carried out over night in 12 ml hybridization mixture (1% SDS,5% Dextran sulphate, 100 μg /ml ssDNA, 0.5 M NaCl) at 65°C. The membranes were washed at 65°C twice for 15 min in 2X SSC (0.01% SDS) and 1XSSC(0.01% SDS). Autoradiography was performed at least over night.

Criteria for defining disease associated alleles

The definition of disease-associated alleles essentially follows the criteria described by us previously (Hu et al. 2003). In summary, sequence variants predicted to result in nonsense mutation or splice-site changes were considered to be disease-associated alleles if they were absent in the DNA of our panel of at least 100 controls. Other variants, such as missense mutations, were considered to be disease associated if they were absent in the same control panel, segregated with the disease in pedigrees, and involved evolutionarily conserved amino acid residues.

Results

Summary of mutation analysis

ABCC6 mutation analysis was carried out in our cohort of 76 PXE patients. 32 patients from our cohort (42.1%) were sporadic cases. Of 42 familial cases, 25 (32.9 %) families had a obvious AR inheritance. Only two families (2.6%) showed a putative dominant inheritance pattern (Plomp et al.2003). No clear segregation pattern in the remaining 17 (22.4%) patients or families was observed.

A summary of the *ABCC6* mutations discovered to date is presented in table 2. A variety of mutations was observed including nonsense, missense, and putative

splice site mutations as well as deletions and one insertion. In our patient cohort, mutant alleles occurred in all combinations including homozygous, heterozygous, compound heterozygous and hemizygous forms. Out of the 152 alleles, 89 (58.6%) exhibited potentially affected alleles, in which 70 (78.7%) could be quickly and efficiently detected by either determining the sizing of PCR products or RFLP analysis on agarose gels. In total, 61 (80.3%) patients were identified with at least one mutanted allele of the *ABCC6* gene.

Phase 1: Rapid detection of the founder mutation R114X and deletion of exons 23-29

The founder mutation, R1141X (Hu et al, 2003b), was found in 35 out of 89 mutant alleles (39.3%). R1141X was the most common PXE mutation in our cohort. The mutation occurred in heterozygous, homozygous and compound heterozygous forms (Table 2) (Figure 1a). This mutation can also be detected by BsiYI restriction digestion and agarose gel electrophoresis and needs to be confirmed by sequencing.

The second most common mutation was a deletion of *ABCC6* exons 23-29. The latter deletion was detected in a multiplex PCR assay using the primers 29a, 29w and 22m (see material and methods). These three primers can amplify both wild type DNA and the exon 23-29 deletion alleles in single PCR reaction. The normal sequence, without the deletion, was amplified by primer set 29a/29w. An allele with the exon 23-29 deletion was detected by the amplification of a fragment of 320 bp generated by the primer combination 29a/w (Fig.1b).

The 23-29 deletion was observed in17 out of 89 mutant alleles (19.1%). The deletion occurred in 14 patients in heterozygous, homozygous, and compound heterozygous forms, and could be detected by PCR amplification and 3% agarose gel electrophoresis (Fig.1b).

Phase 2: Rapid detection of a core set of mutations

After screening of R1141X and del exons 23-29, a core set of nine different mutations was rapidly identified by PCR and PCR-RFLP. We detected this set of core mutations in 18 alleles (20.2%) in three subsequent steps: First, the 1944 del22 in exon 16, and 3821 del48 in exon 27 were identified after PCR and gel electrophoresis by size differences between the wild type and mutant alleles (Fig.2a). These different mutations presented in heterozygous or compound heterozygous form in three patients with PXE in our cohort. Next, R765Q, G1302R, A1303P,

3775 del T, and R1459C mutations were detected in 15 mutant alleles by digestion of PCR fragments with restriction enzymes SmaI, NciI, HaeIII, BstNI, and AciI, respectively. After 3% agarose gel size segregation, cleaved or uncut fragments represented wild type or mutant forms could easily be interpreted (Fig 2b, 2c, 2d).

By the methods described in phases 1 and 2, 70 out of 89 affected alleles (78.7%) were detected. Further mutation analysis was carried out by DHPLC.

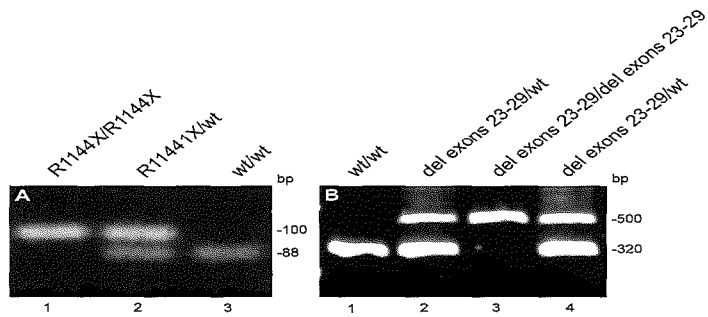

Figure 1:

ABCC6 mutation analysis, Phase I: Detection of the R1141X mutation in exon 24 and deletion of exons 23-29.

(A) BsiYI-digested PCR products of exon 24 were size fractioned on 3% agarose gel. Lane 1: The R1141X mutation disrupts the BsiYI site in the wild type sequence, and results in a single PCR fragment of 100 bp. Lane 2: The wild type allele was cleaved into two BsiYI fragments of 88 bp and 12bp (latter band not visible).

(B) Detection of ABCC6 deletion of exons 23-29 by multiplex PCR using 3 primers. A detailed explanation of this test is given in the text. Lane 1: a 320bp PCR product from wild type; Lane 3: mutant allele with deletion of exons 23-29 yields a 500bp fragment; Lane 2, 4: Amplification fragments from heterozygotes for the exons 23-29 deletion. The genotypes are indicated above each sample lane.

Table 1
Primers for mutation screening of the ABCC6 gene

Exon	sense primer (5'-3')	antisense primer(5'-3')	*size (bp)	PCR (Tm)	#DHPLC
1	tgctgggtccaaagtgttta	cagcccgagagatctgcagc	469	57	62.1
2	gatccaaaaagttgtcctggc	tgtcccctgcctcccccgaa	328	57	62.5
3	ttgttctccactgtggcagg	ctgtgacctctcttattgccc	246	55	61.5
4	gagccaccattttggtttccc	cctgccacagtggagaacaa	263	55	62.5
5	cctctgtctccattccttat	agactgagacctcaaagtgg	220	57	60.5
6	cacagttcgtcctgtcttcc	ggccctggagaagcagctgt	620	57	58.1
7	gatcctgcaggggtgaatgg	atgatgagcttttctgaagt	242	57	61.7
8	cccccaactcccatgattgc	aaggatgccactaagagacc	450	57	60.6
9	tcagtgatactgcttttcct	gcacctcctctcaccagc	280	55	61.6
10	actccgttcaaatcccgtct	gccccggcctccccacttta	244	57	63.0
11	ctgtggcttcctccctactt	ttcacgtgcctctgagagct	211	57	61.0
12	gtgctgctcagcatagagac	cagggtgcagggaagaattc	302	57	60.5
13	gaagctggagccaggtgtag	tcactgttgccccacgtgtc	250	60	62.9
14	gatgctggcttgccattatgg	tgctcttcttgctgggtgac	213	57	63.5
15	aggagccccatgcatcttct	gtaaatggcctcttctgccc	160	57	61.7
16	gacatcctagcagacaggct	aaccaaggtcatgtctccc	196	57	63.3
17	atgatgagtcggggacccaa	ccatcatcctcctgtgacca	288	57	62.1
18	gctaagtgcttcctctgcct	caccctgtacatgctgagtc	280	57	61.2
19	tggccagagcactccattca	ccggccacatgtgtgtaact	315	57	62.2
20	cagcttcagcctgtgcccttt	gggtgtcaaagtgggtatctg	183	60	63.5
21	ctgccaagtgctacatttgg	ccattgggagagatactgac	254	57	61.3
22	ttggtgcagctgggaggaga	ctctccctcatgtgtgctac	280	57	64.5
23	gactggctgagttgacctcag	ggtctccagccctcatgctctt	408	60	63.1
24	aaggtcttctctgccctggctctt	cttccctctcccatccatccttct	256	57	64.4
25	agtctctgcctctgtctgtc	gagagtaaccactcaccctg	220	57	63.4
26	agcagatgtcaacagggacc	tgttgcaagccctcaagtgg	240	57	63.5
27	cctggagtcctttggcctaa	ctgactcagtttcccctcct	230	57	61.2
28	cccaccatgcctcccatctt	gcccacaaaccctctggtca	254	57	65.1
29	ccatggtgggacgaccatac	aagatgggaggcatggtggg	240	57	65.2
30	agctctaacccgaagcccag	catgtgccgacaaacgcacg	341	57	63.8
31	tccaactggggtacggttga	agacacactgggctctcaca	219	57	64.1

** Size amplified fragment; # Optimized temprature of each fragment for DHPLC.*

Table 2
Results of the mutation analysis in the ABCC6 gene in 76 patients

type	AA change	Sequence variation	location	alleles	*status	#phase	method
Nonsense							
	Q749X	2247 C >T	Exon 17	2	ht	3	DHPLC
	R1141X	3421C > T	Exon 24	35	hm.ht.ch	1	BsiYI
Missense							
	R765Q	2294G > A	Exon 18	1	ht	2	SmaI
	R1114H	3341G > A	Exon 24	1	ht	3	DHPLC
	T1130M	3390C > T	Exon 24	2	ch	3	DHPLC
	R1221C	3663C > T	Exon 26	1	ch	3	DHPLC
	G1302R	3904G > A	Exon 28	1	ht	2	NciI
	A1303P	3907G > C	Exon 28	1	ch	2	HaeIII
	D1326N	3999G>A	Exon 28	1	ht	3	DHPLC
	K1394N	4182G > T	Exon 29	1	hm	3	DHPLC
	R1459C	4377C > T	Exon 30	1	ht	2	AciI
Frameshift							
Splicing							
		IVS17-12delTT	Intron 17	1	ht	3	DHPLC
		IVS26-1G>A	Intron 26	1	ht	3	DHPLC
Deletion							
		1944del22	Exon 16	2	ht,ch	2	PCR
		4182delG	Exon 29	6	hm,ht	3	DHPLC
		3775delT	Exon 27	11	hm,ht	2	BstNI
		3821del48	Exon 27	1	ht	2	PCR
Insertion							
		4220insAGAA	Exon 30	1	ht	3	DHPLC
Deletion	A995del405	del exon23-29	Exon23-29	17	hm,ht,ch, hem	1	PCR
			ABCC6	2	ht	4	FISH

*hm-homozygote;ht-heterozygote;ch-compound heterozygote;hem-hemizygote.

phase of detection protocol.

Phase 3: Detection of additional mutations by DHPLC

DHPLC analysis through complete coding sequence was performed for patients who showed one or no disease-causing mutation after detection by the first two phases. Ten additional known nucleotide changes, including Q749X, R1114H, T1130M, R1221C, D1326N, K1394N, 4182delG, IVS17-12delTT, IVS26-1G>A, and 4220ins AGAA were successfully identified by DHPLC under the conditions presented in table 1 (Figure 3). These mutations occurred on 17 additional alleles (19.1%) of the *ABCC6* gene in the group of PXE patients.

Phase 4: Mutation detection by Southern blot or FISH

In the DNA of 10 additional patients we could not find any mutation with the strategies described above. In two of these patients, we found large deletions. One encompassed the entire *ABCC6, ABCC1* and *MYH11* genes. In the other patient the deletion included at least the entire *ABCC6* gene (Hu et al. 2003a).

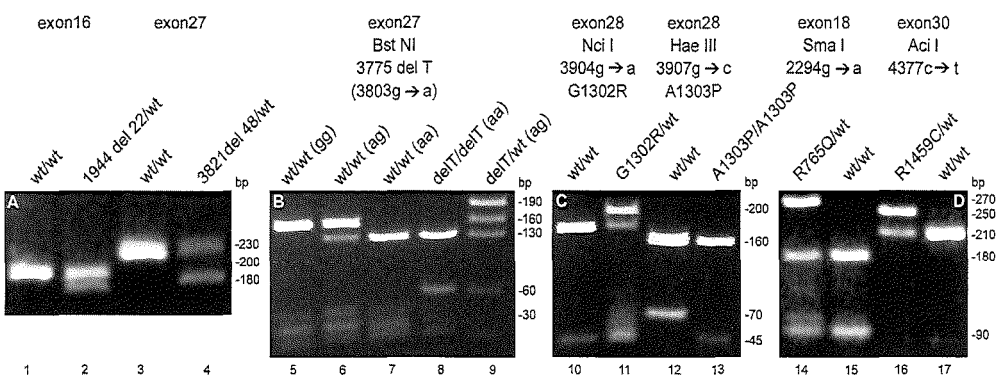

Figure 2 ABCC6 mutation analysis, phase II: Identification of seven mutations by PCR-RFLP analyses. The genotypes are indicated above each sample lane.
(A) PCR fragments containing: Lane 1: exon 16 wild type (196bp); Lane 2: exon 16 wild type (upper band) and exon 16 with a 22bp deletion (lower band); Lane 3, exon 27 wild type (230bp); Lane 4: exon 27 wild type (upper band), and same fragment with a 48bp deletion (lower band).

Figure 2 continued: (B): PCR fragments of exon 27 were digested by BstNI. This enzyme cleaves the PCR fragment potentially twice: The BstNI enzyme not only cuts the sequence if a T is present at position 3775 (wild type), but also if a G (wild type) is replaced by an A (polymorphism) at position 3803 (3803 G>A, shown in brackets). Lane 5: 3775 T (wild type) and 3803 G (wild type) produced two expected BstNI fragments of 162bp and 33bp; Lane 6: 3775 T (wild type) and 3803 G or A (heterozygote for polymorphism) yields indeed fragments of 162bp, 132bp, and 33bp; Lane 7: 3775 T (wild type) and 3803 A showed products at size 132bp and 33bp; (Lane 8): Deletion T at 3775 leads to the loss of one BstNI site, combining with 3803A produced fragments of a 132bp and a 63bp; Lane 9, One allele presented T and another allele lost T at 3775 combined G or A at 3803 resulted in 195, 162,132 and 63bp.

Note that the amplification pattern in lane 9 originated from a compound heterozygote at both nucleotide 3775 and 3803: Either 3775T/3803G (allele 1) and 3775delT/3803A (allele 2) or 3775T/3803A (allele 1) and 3775delT/3803G (allele 2). Assuming the first genotype, four of fragments, 162, 132, 63, and 30bp, would have to be generated; However, the latter genotype would produce fragments of 195, 132, 33, and 30bp. The two extra, non-original allelic sequences seen in lane 9 may result from the formation of template heteroduplexes during PCR amplification, as previously described (Ayliffe MA et al, 1994; Jansen R and Ledley FD, 1990). The specific genotype (del T/wt; A/G) associated with the amplification pattern in lane 9 was confirmed by sequencing the amplification product in at least 5 unrelated individuals. Furthermore, note the presence of primer-dimers in lanes 5-9 around the 30 bp.

(C) PCR fragments of exon 28 were digested by NciI. Lane 10: Wild type fragment (3904 G) was cut into three fragments, 180, 55, and 20bp (latter band not visible); Lane 11: In the mutant allele (3904 A), a NciI site is abolished which yields one additional NciI fragment of 200bp in the heterozygote; Lane 12: PCR fragments of exon 28 were digested with HaeIII, which cuts the wild type sequence in fragments of 160bp and 70bp; (Lane 13) a C instead of G at position 3907 on the mutant allele in exon 28 resulted in one additional HaeIII site and yielded fragments of 160bp, 45bp, 25bp (latter band not visible).

(D) A PCR fragment of exon 18 was digested with SmaI. SmaI cuts the sequence only if a G is present at position 2294 (wild type) and yields 180bp and 90bp bands (Lane 15). A mutation at 2294 (G→A) yields an additional fragment of 270bp. (lane 14) The light band seen in lane 14 at approximately 100 bp is a PCR artefact. Lane 16 and 17: PCR fragments of exon 30 were digested by AciI. The wild type allele with a C at position 4377 in exon 30 was cleaved into two fragments of 210 bp and 40 bp (latter band not shown); The mutant allele with a T at 4377 disrupted a cut site of AciI and produced only one fragment of 250bp. In lane 16, a heterozygote, and in lane 17 a homozygote wild type, are shown.

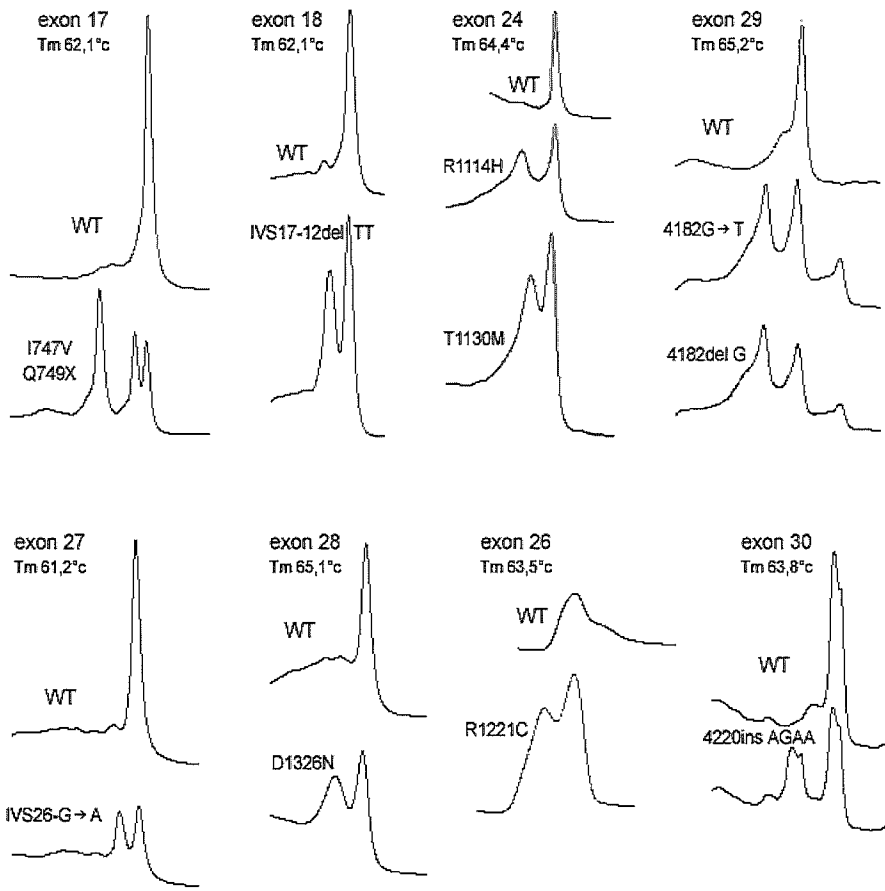

Figure 3: Mutation detection using positive controls run on DHPLC in the *ABCC6* gene. The mutation type and the temperature running on DHPLC are indicated by the specific elution profile. The wild type elution profile is given on the top. The abnormal profile of mutation containing PCR fragments are given directly below wild type profile. Primers used are presented in Table 1. A more detailed description of each mutation is given in Table 2.

Discussion

Efficient and quick molecular diagnosis of PXE

We have carried out *ABCC6* DNA diagnostics in 76 PXE patients. We screened 152 ABCC6 alleles which led to mutation detection in 61 cases (80.3%) and 89 disease associated alleles (58.6%). Our diagnostic strategy is a medium throughput strategy. As frequently seen in mutation analysis of disease genes, a few (*ABCC6*) mutations are common, while the majority (in the *ABCC6* gene) is unique to a single case or family. The ideal screening strategy should be technically simple, cost-effective, sensitive and enabling efficient identification of known and novel unique mutations.

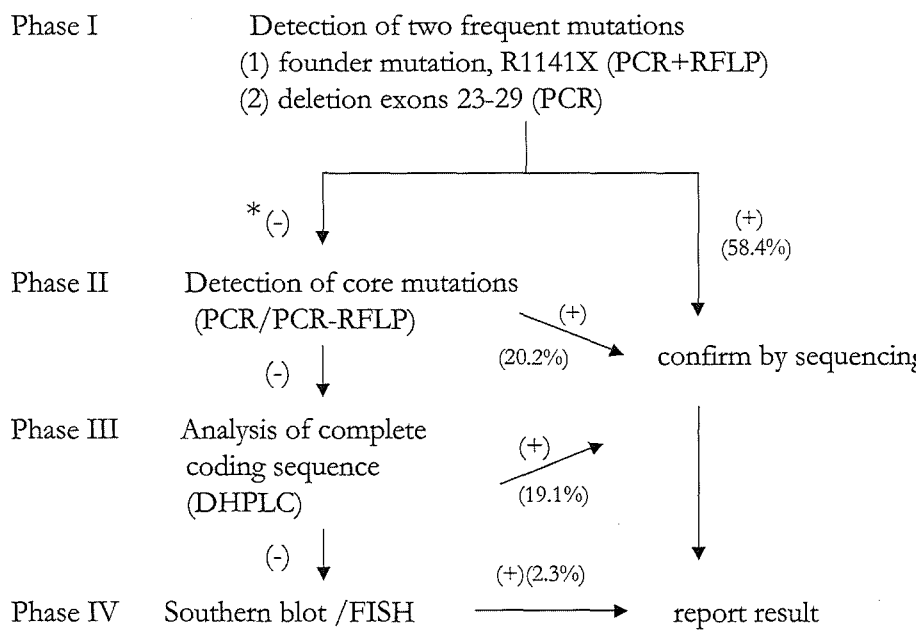

Figure 4: Scheme of the screening strategy which is divided in four subsequent phases (phase I – IV). *(-),(+) indicate negative and positive results. Percentage (%) presented is alleles detected in each phase divided by total mutant alleles.

Our strategy described here enables a simplified screening procedure starting with PCR and PCR-RFLP (Figure 4). In two first steps, nine mutations including two frequent ones were identified efficiently by these fundamental methods. The latter means that 78.7% out of all known mutant alleles can be screened and confirmed for each patient in a single week. Next, extensive screening of the ABCC6 coding sequence was necessary for patients with one or no detected mutant allele. For this purpose, we used the advanced DHPLC method. DHPLC has the capability to analyse 100 specimens around ten hours. Compared with SSCP, dHPLC is approximately six times faster and three times less costly.per sample analysed. The additional mutations identified in our patient's cohort by DHPLC constituted for 19.1% of total mutations.

Together, the combination of basic PCR, PCR-RFLP methods with advanced DHPLC analysis seems the most attractive for rapid and efficient molecular diagnostics in PXE patients.

The detection rate in our group was comparable to another study of a European PXE cohort of 61 cases (Le Saux et al. 2001). However, it appears that a large number of mutations, if present at all, still remain undetected. We previously suggested that this may be due to genetic heterogeneity, expression of PXE features in individuals carrying only a single mutation, clinical misdiagnosis, the presence of yet undetected (large) deletions, the presence of mutations in introns or the promoter region or may be due to misdetection because of pseudo gene homologies. Furthermore, we may have missed intragenic deletions using Southern blot because of cross-hybridisation with putative pseudogene sequences. In conclusion, clinical re-examination and FISH may be recommended for those patients in our cohort for whom no ABCC6 mutation was detected yet.

Application of DHPLC in mutation detection of the ABCC6 gene

DHPLC is a relatively novel mutation detection method and was originally used to identify single nucleotide polymorphisms on the Y chromosome (Underhill et al. 1996). It is a semi-automated technology for mutation screening based on the separation of heteroduplexes and homoduplexes on a stationary phase under partially denaturing condition. Compared with SSCP, mutation screening by DHPLC is more efficient and detects up to 98% of all mutations in some disease causing genes, such BRCA1 and BRCA2 (Wagner et al. 1999). As this is the first application of DHPLC in the mutation detection of the *ABCC6* gene, we

optimised the usefulness of this method for *ABCC6* analysis. To evaluate the sensitivity, a set of previously known samples with *ABCC6* mutations were tested on DHPLC. The results of DHPLC testing were concordant with the SSCP and direct sequencing. In addition, two additional novel mutant alleles (IVS 26-1 g>a, D1326N), which were missed by SSCP analysis previously, were identified.

Through control sequencing, we noted false positive profile signals by DHPLC in nine out of 104 fragments (8.7%; data not shown). In our experience, the latter is probably due to the poor quality of PCR fragments or due to mutations which are introduced during PCR. These problems can be avoided by optimising the PCR conditions and the use of re-amplified PCR products in control experiments.

In summary, in cases of known mutations, DHPLC signature chromatogram analysis is more efficient than the traditionally used time-consuming and laborious SSCP and sequencing methods. However, in samples with yet unknown mutations, avoiding false positive signals and differentiating between missense mutations and polymorphisms remains a challenge.

Molecular diagnostics of PXE: consequences for genetic counselling

The clinical expression of PXE is heterogeneous with considerable variation in phenotype within and between families. No international consensus or 'gold standard' exists to establish the clinical diagnosis of PXE. A thorough clinical investigation of all family members as well as extensive mutation screening for mutations on both *ABCC6* alleles remains essential for molecular diagnosis and genetic counselling of PXE patients and families.

Even if one or two *ABCC6* two mutations are known, the interpretation of the molecular diagnosis for PXE patients for genetic counselling remains difficult. First of all, the majority of patients with PXE are sporadic. Next, in familial cases, the inheritance mode of PXE is incompletely understood. In the vast majority of families, an AR inheritance pattern is observed, with sometimes slight penetrance of clinical features in carriers (Bacchelli et al. 1999, Sherer et al. 2001). In addition, in about 2% of familial cases autosomal dominant (AD) inheritance might be assumed according to our latest data (Plomp et al, unpublished data). Our group investigated one PXE patient, from a putative dominant family, with a single missense mutation (R1459C) in detail. In an effort to detect a pathogenic mutation on the second allele, we sequenced all exons of ABCC6, 1 kb putative promoter region, and the full length ABCC6 mRNA. Despite this extensive effort, no

second mutation was found. Haplotype analysis in the family, using markers within and flanking the ABCC6 gene, did not yield evidence for the presence of a (large) deletion (Hu et al. 2003a, Plomp et al., in press). To fully elucidate the inheritance pattern and molecular etiology of PXE in this family, the functional consequences of this mutation remains to be studied in patient fibroblasts and/or cell lines.

In summary, our strategy can be used as a rapid and efficient approach to screen for mutations in the *ABCC6* gene and improves molecular diagnosis for PXE patients. Extensive and careful clinical and molecular investigation of newly diagnosed PXE patients and families may be helpful for genetic counselling and will proved further insight into the molecular pathology of PXE.

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Chapter 5

Frequent mutation in the ABCC6 gene (R1141X) is associated with a strong increase in the prevalence of coronary artery disease

Abstract

Background: Pseudoxanthoma Elasticum (PXE) is an inborn disorder of the connective tissue with specific skin, ocular and cardiovascular disease (CVD) manifestations. Recently, we and others have identified mutations in the gene coding for the ABCC6 transporter in PXE patients with ocular and skin involvement. In the Netherlands, as in the rest of Europe, a particular premature truncation variant ABCC6 (R1141X) was found in a large cohort of PXE patients. Given the association between CVD and PXE we hypothesized that heterozygosity of this ABCC6 mutation could also confer an increased risk for CVD.

Methods and results: To assess the relationship between the frequent R1141X mutation in the ABCC6 gene and the prevalence of premature coronary artery disease (CAD), we conducted a case-control study of 441 patients under the age of 50 years with definite CAD and 1057 age- and sex-matched population-based controls who were free of coronary disease. Strikingly, the prevalence of the R1141X mutation was 4.2 times higher among patients than among controls (3.2% vs. 0.8%; $p < 0.001$). Consequently, among subjects with the R1141X mutation, the odds ratio for a coronary event was 4.23 (95% CI: 1.76 – 10.20, p=0.001).

Conclusion: The presence of the R1141X mutation in the ABCC6 gene is associated with a sharply increased risk of premature CAD.

Introduction

Pseudoxanthoma Elasticum (PXE) is an inborn disorder, the hallmark of which is dystrophic mineralization of elastic tissue of the skin, retina and arterial walls.[1-4] Most PXE patients seem random, but autosomal recessive and autosomal dominant inheritance also is observed.[5,6] The frequency of PXE in the general population is unknown, particularly because it is likely that individuals with a mild clinical phenotype will escape diagnosis. Recently, we and others elucidated the molecular basis of PXE by demonstrating mutations in an ATP- binding cassette (ABC) transporter gene (ABCC6) as the cause for this disorder.[7-12] Cardiovascular manifestations of PXE include accelerated atherosclerosis, which results in myocardial infarction at a young age, attributed to calcification of the internal elastic laminae of the coronary arteries. On several occasions we were struck by the

fact that, in our patients suffering from premature cardiovascular disease, PXE was found to be concomitantly present. Whether carriership of a single ABCC6 gene mutation on one allele would also confer additional risk for CAD was hitherto impossible to assess. This situation changed with the elucidation of the molecular basis of PXE. In parallel studies (Xiaofeng Hu, unpublished data), we found that the R1141X mutation is the most common mutation in the Dutch PXE patients and families, and it seems as though this is the case for the rest of Europe as well. We therefore studied the prevalence of the R1141X mutation in the ABCC6 gene in patients with premature CAD and in a large population based group of healthy controls to further delineate the role of this genetic variation as a risk factor for CAD.

Methods

Case and Control Population

Consecutive Dutch patients under the age of 50 years with CAD (n = 441) referred between 1995 and 2001 to the Atherosclerosis Outpatient Clinic of the Academic Medical Center of the University of Amsterdam were included in the study. Patients qualified for inclusion after a myocardial infarction, surgical or percutaneous coronary revascularisation, or a coronary angiogram with evidence of at least a 70% stenosis in a major epicardial artery. The Institutional Review Board approved the protocol. All patients gave informed consent.

Control subjects (n = 1057) were selected from the participants of the Cardiovascular Disease Risk Factor Monitoring Project, a large project that screened for cardiovascular risk factors and was carried out in three Dutch towns (Amsterdam, Doetinchem and Maastricht) between 1987 and 1991. All participants completed an informed consent form, agreeing to the use of stored blood samples for further scientific research. A detailed description of these examinations is previously published.[13] Approximately 2 controls per case were selected, group matched for sex and age (within 5 years). All controls were Dutch and reported no history of myocardial infarction, percutaneous transluminal angioplasty, or coronary artery bypass grafting in a self-administered questionnaire.

Mutation analysis:

Genomic DNA was extracted according to standard protocols. The polymerase chain reaction primers used to amplify exon 24 were MRP6 ex24F: aaggtcttctctgccctggctctt ; ex 24R :cttccctctcccatccatccttct.

After PCR (20 ng/ µl DNA in 25 µl) the product and an internal control were digested with the restriction enzyme BsiY1. Mutated products remain uncut. The fragments obtained were separated on a 3% agarose gel and visualized after staining with ethidium bromide. The presence of the mutation was confirmed by direct sequencing.

Biochemical analysis:

For the CAD patients the plasma cholesterol and triglycerides were determined with commercially available enzymatic methods (Boehringer Mannheim, FRG, Nos.237574, and Sera-PAK, Miles, Italy, no 6639, respectively). To determine high-density lipoprotein cholesterol, the polyethylene glycol 6000 precipitation method was used. Low-density lipoprotein cholesterol was calculated by the Friedewald formula. The biochemical analysis for the controls is described previously.[13]

Statistical analysis:

Fisher's exact test was applied to compare allele frequencies between groups, and exact 95% confidence intervals were calculated for the odds ratio, with adjustment for matching criteria. Risk factors were compared between cases and controls, and between carriers and non-carriers using Fisher's exact or *t*-test, where appropriate.

Results

The characteristics of the 441 cases and 1057 controls are presented in Table 1. As expected, the frequency of increased body mass index, dyslipidemia, smoking, hypertension and diabetes was increased in cases versus controls. In cases, 14 out of 441 (3.2%, 95% CI: 1.9-5.6%) were carriers of the R1141X truncation variant, whereas 8 out of 1057 controls (0.8%, 95% CI: 0.3-1.5%) carried this ABCC6 mutation, yielding a statistically significant difference at a probability value of <0.001 with an odds ratio corrected for age and sex of 4.23 (95% CI: 1.76 to 10.20).

Table 1. Characteristics of cases and controls

Characteristic	Cases n = 441	Controls n = 1057	p-value
ABCC6 mutation carriers	14 (3.2%)	8 (0.8%)	0.001
Male sex	358 (81%)	801 (76%)	0.029
Age, years	40.4 ± 6.2	39.4 ± 6.9	0.006
BMI, kg/m^2	27.0 ± 4.0	25.3 ± 3.8	<.001
Total cholesterol (mmol/l)	5.83 ± 1.54	5.39 ± 1.04	<.001
HDL-cholesterol (mmol/l)	1.10 ± 0.31	1.22 ± 0.33	<.001
Smokers	320 (73%)	390 (37%)	< 0.001
Hypertension	168 (38%)	158 (15%)	<0.001
DM	125 (28%)	6 (0.6%)	<0.001
History of MI	323 (78%)	-	

Values are mean ± SD or n (%). BMI= body mass index; DM= diabetes mellitus; MI= myocardial infarction.

Table 2.
Baseline characteristics of patients according to R1141X genotype

Variable	Heterozygous N = 14	Wild type N = 427	p-value
Age years	48.1 ± 6.0	47.3 ± 6.0	0.64
Male sex,	10 (71%)	322 (80%)	0.43
Age at diagnosis	39.6 ± 6.9	40.6 ± 6.1	0.56
History of MI	11 (79%)	312 (78%)	0.93
Smoking	4 (29%)	113 (28%)	0.84
BMI, kg/m^2	26.4 ± 3.6	26.8 ± 4.0	0.71
Hypertension	8 (57%)	145 (36%)	0.13
DM II	6 (43%)	111 (27%)	0.13
Family history CAD	9 (64%)	229 (57%)	0.33
Total cholesterol, mmol/l	5.96 ± 1.36	5.81 ± 1.60	0.73
HDL-cholesterol, mmol/l	1.01 ± 0.17	1.10 ± 0.31	0.29
LDL-cholesterol, mmol/l	3.96 ± 1.27	3.82 ± 1.53	0.74
Triglycerides, mmol/l	2.19 ± 1.43	2.09 ± 2.12	0.43

Values are mean ± SD or n (%). MI indicates myocardial infarction; CAD, coronary artery disease; BMI, body mass index; DM, diabetes mellitus; HDL-cholesterol, high density lipoprotein cholesterol; LDL-cholesterol, low density lipoprotein cholesterol.

We subsequently categorized the premature CAD patients in carriers (n = 14) and non-carriers (n = 427) of the R1141X variant of the ABCC6 gene (Table 2). The major risk factors for CAD were equally divided in both groups.

Discussion

We demonstrate in a large case-control study that a strong association exists between a frequent mutation in the ABCC6 gene (R1141X) and the presence of premature CAD. Carriers of this mutation had an odds ratio of 4.2 for CAD when compared with non-carriers. In addition, we could not find a relation between this mutation and other major CAD risk factors, suggesting that this mutation in the ABCC6 transporter is operating through a novel pathway in atherogenesis.

PXE is characterized by deranged elastic fiber metabolism, resulting in fragmentation and calcification of elastic fibres, with resultant changes in the skin, eyes, gastrointestinal tract and cardiovascular system. Cardiovascular manifestations in PXE include premature CAD, cerebrovascular disease, peripheral vascular disease and renovascular hypertension. Calcium deposits in the elastic lamina of the arterial wall indeed resemble the other calcium deposits seen in PXE patients.

PXE-like elastic tissue disorders have also been documented in sickle cell disease, β-thalassemia and sickle thalassemia, in Marfan's syndrome, Ehlers-Danlos's syndrome and Paget's disease.[14] The pathology of these PXE-like syndromes is generally considered to be one of the manifestations of the underlying systemic illness. PXE, or at least a number of its clinical manifestations, could therefore also be considered as secondary to an underlying systemic disorder.[15]

Recently, mutations in the ABCC6 gene have been established as the cause of PXE. The exact biological function of ABCC6, however, is presently still unknown, as is the functional relationship of this transmembrane transporter to the pathogenesis of the PXE phenotype. ABCC6 messenger-RNA was reported to be highly expressed in the liver and kidney in contrast to tissues characteristically affected by PXE.[16]

Whatever the specific pathophysiology of PXE, our study results seem to indicate that mutations in the ABCC6 gene are not rare in the general population and contribute to an increased propensity towards premature atherosclerotic vascular disease. If our data are subsequently confirmed in other cohorts, this

might have implications for genetic screening in PXE kindreds and may require a more aggressive approach towards CAD prevention in these individuals.

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Chapter 6

Does Autosomal Dominant Pseudoxanthoma Elasticum Exist?

Abstract

Pseudoxanthoma elasticum (PXE) is a progressive disorder of elastic fibres in skin, eyes and arterial walls. It is caused by mutations in the *ABCC6* gene. Most patients are sporadic cases. The majority of familial cases show autosomal recessive (AR) inheritance, but autosomal dominant (AD) inheritance has also been reported. We reviewed the literature on AD PXE and we studied in detail, both clinically and by DNA studies, a selection of potentially AD pedigrees from our patient population consisting of 59 probands and their family members. Individuals were considered to have definite PXE if they had two of the following three criteria: characteristic ophthalmologic signs, characteristic dermatologic signs and a positive skin biopsy. In the literature we found only three families with definite PXE in two successive generations and no families with definite PXE in three or more generations. Our own data set comprised three putative AD families. Extensive DNA studies revealed a mutation in only one *ABCC6* allele in the patients of these families. Only one of our families showed definite PXE in two generations. Linkage studies revealed that pseudodominance was unlikely in this family. In the other two families AD PXE could not be confirmed after extensive clinical examinations and application of our criteria, since definite PXE was not present in two or more generations.

Conclusion: the inheritance pattern in PXE usually is AR. AR PXE carriers may show mild features of the disease. Autosomal dominant inheritance in PXE may exist, but is, both after careful literature study and in our patient material, much more rare than previously thought.

Introduction

Pseudoxanthoma elasticum (PXE) is a heritable disease of elastic tissue, especially affecting the skin, the eyes and the cardiovascular system. The prevalence is estimated to be about 1 in 100.000. The skin shows yellowish papules and plaques, mainly on the lateral side of the neck and on flexural areas of the body, sometimes accompanied by redundant skin folds. Common ocular signs are peau d'orange, followed by angioid streaks, of which the complications can eventually lead to severe visual loss. Patients have an increased risk of cardiovascular disease and of (mainly gastrointestinal) hemorrhages. Histopathologically, elastic fibers in the

affected tissues show rather characteristic fragmentation, clumping and calcification.[29]

At a consensus conference in 1992 diagnostic criteria for PXE have been defined.[25] Major criteria were "characteristic skin involvement", "characteristic histopathologic features of lesional skin" and "characteristic ocular disease in adults older than 20 years of age". Minor criteria were "characteristic histopathologic features of non-lesional skin" and "family history of PXE in first-degree relatives". Based on these criteria five different PXE categories were distinguished. Unfortunately, minimal diagnostic criteria for the diagnosis "PXE" have not yet been established. There are no pathognomonic clinical signs, apart from comet-like lesions in the retina.[16]

Establishing the inheritance pattern in PXE pedigrees solely on the basis of clinical data is difficult and complicated by the variable expression of the disease, the presence of mild symptoms in heterozygous individuals, mimicking dermatoses, as well as potential pseudodominance due to consanguinity or high carrier frequency. The majority of PXE cases is sporadic.[41] In families, autosomal recessive (AR) inheritance was mostly observed, but a small subset of families was reported to have autosomal dominant (AD) inheritance. However, even when PXE symptoms are present in two subsequent generations, AD inheritance remains uncertain.[29,42] Recently, the gene for PXE, ABCC6, was identified.[4,24,35] Mutations in ABCC6 have been found in sporadic patients, in families with AR as well as in families with possible AD PXE. There are no indications for genetic heterogeneity of the disease.[40,23]

Recently, the gene for PXE, *ABCC6*, was identified.[4,24,35] Mutations in *ABCC6* have been found in sporadic patients, in families with AR as well as in families with reported AD PXE. There were no indications for genetic heterogeneity of the disease.[40,23] Obviously, if both *ABCC6* alleles of a patient carry a mutation, AR inheritance is most plausible in that family. However, if only one mutation is found, the presence of a second, as yet unknown, mutation can not be excluded. The current mutation detection rate for *ABCC6* mutations implicated in PXE was at least 0.55 (mutations per allele). In 22 (37%) of 59 patients a mutation was found in both alleles.[17] For genetic counselling it is important to know if AD inheritance really exists in PXE and, if so, what its frequency and penetrance are. The aim of this paper was to scrutinize the existing literature on evidence for AD PXE according to present standards and to study our data set of 59 PXE patients and families, both clinically and by DNA studies, for evidence of AD inheritance.

Material and methods

Literature search

A PubMed search of relevant literature spanning the period 1966 to February 2002 was performed using search terms "pseudoxanthoma elasticum" and "dominant". Further articles were found in the reference lists of other articles. For each published pedigree, the size and structure of the families, age of the family members, and the reported skin and eye abnormalities for each family member were reviewed. Individuals were considered to have definite PXE if they had at least two of the three following criteria: ophthalmologic or dermatologic signs or a positive skin biopsy, as mentioned below, even if not reported in detail (like 'classical' or 'typical' skin abnormalities). If there was uncertainty about one of two present criteria, the diagnosis was considered probable.

Clinical examination of patients

All 23 patients and family members from the three families, which participated in this study, were examined by an ophthalmologist and dermatologist. Ophthalmologic examination included assessment of visual acuity, slit-lamp examination, fundoscopy and, in case of doubt, fluorescein angiography. The majority of the participants (15/23) had a skin biopsy. The ophthalmologist, dermatologist and pathologist were masked as to the genotype of the patients. Blood was taken for DNA studies. Permission for this was given by the medical ethical committee of the Academic Medical Center in Amsterdam and informed consent was obtained. We considered the diagnosis PXE definite if two of the following three criteria were present: 1. yellowish papules and/or plaques on the lateral side of the neck and/or flexural areas of the body (especially the axillae, antecubital fossae, groins and popliteal spaces), 2. typical histopathological changes in a skin biopsy after Von Kossa staining (fragmentation, clumping and calcification of elastic fibers), 3. one or more of the following retinal abnormalities (seen at any time during the patients life): peau d'orange, angioid streaks or comet-like streaks (pinpoint white lesions of the choroid with a hypopigmented tail in the retinal pigment epithelium, also called "comets".[16]

Molecular analysis

Isolation of DNA from peripheral blood samples and haplotype analysis with microsatellite DNA markers was performed in families according to standard protocols essentially described elsewhere.[43] PCR primers were selected from the published sequence of human chromosome 16 BAC clone A-962B4 (GenBank Accession No. U91318), TIGR database (http://www.tigr.org), or the primers were a gift of collaborators (C. Boyd). To distinguish between the ABCC6 gene and pseudogene sequences, novel primers for exon 1-9 were developed.[34] To amplify and screen both exon and the adjacent intron sequences, PCR products were derived from intronic sequences 20-50 bp out from the end of each ABCC6 exon. PCR was performed on DNA in each PXE patient. PCR products were pre-screened using single strand conformational polymorphism (SSCP) and denaturing high performance liquid chromatography (DHPLC). Fragments with a mobility shift were characterized by direct sequencing.[4] All putative disease causing mutations were also screened in at least 100 control chromosomes from healthy (ophthalmologically examined) individuals from a hospital based Dutch population, to distinguish the disease causing mutations from polymorphic variants. The potential presence of intragenic large deletions of genomic DNA was confirmed by consistent lack of amplification of the relevant exons in patients who were heterozygous or homozygous for the deletion. Intragenic deletions were detected by FISH or Southern blots using PCR-amplified ABCC6 exons as a probe.

Results

Literature on autosomal dominant PXE families

Pope previously observed a frequency of AD PXE of 53%.[32] He reported to have clinic genetic data on 142 patients. There were 121 index patients and families of which 64 were classified as AD, and the remainder as AR. The patients from families with multiple affected generations and with all possible combinations of parent-child transmission were placed in the AD group. The families with affected sibs but no affected parents or children were placed in the AR group. Based on clinical differences alone he distinguished two AR and two AD types.[33] Sporadic cases were allocated to one of these types based on clinical findings. On the other hand, Neldner found potential AD inheritance in only three (3%) out of a population of 100 PXE patients. Two of these three families comprised a mother-daughter pair, the third patient was said to have a father with PXE. No further details were given.[29]

We selected 18 putative dominant PXE families from the literature, which were described in 16 publications. A summary of the data is presented in table 1. In most reported 'dominant' families no definite diagnosis PXE could be made in two (or more) generations, on the basis of our criteria.[3,8-13,14,19,21,22,30,44] Only in a minority of patients a skin biopsy was reported.

On the basis of our criteria, only three families, in three different reports, presented with PXE in two successive generations.[1,7,32] In figure 1 a review of these pedigrees is presented, adapted to our criteria. Interestingly, we did not find a single pedigree with definite PXE in three or more generations.

In addition to the families presented above, our search yielded the following reports on AD PXE, that we excluded for various reasons: A male proband with characteristic skin lesions, a positive biopsy, angioid streaks and retinal hemorrhages had a maternal aunt, who also complained of poor vision and had a cutaneous condition similar to his. No more details were given.[20] A mother and her three children did have typical skin abnormalities, but no ophthalmologic signs. The mother had married her cousin, so that AR inheritance is most likely.[27] A father with PXE had a son with probable PXE, but no further details were given.[14] In yet three other patients, who were said to have AD PXE type I, the microscopic and biomechanic features of skin were studied, but the families of these patients were not described.[31] In four recently described families, in which the children were diagnosed with PXE, one of the parents appeared to have limited phenotypic expression.[38] Molecular studies had not been performed yet. The authors concluded that the inheritance pattern in these families was not clear. In a short report a female proband with PXE is described.[26] The authors only mentioned briefly that several other family members were affected, in accordance with AD inheritance.

Clinical and molecular results in our putative AD families

We investigated and collected data from 59 apparently unrelated PXE probands from the Netherlands and their family members. In 41,9% of the families PXE segregated in a clear-cut AR fashion. Up to 53 % of the patients were sporadic cases or the familial segregation pattern was not clear. The only three families (5%), in which there was a putative AD inheritance pattern, were investigated thoroughly and are described in detail here.

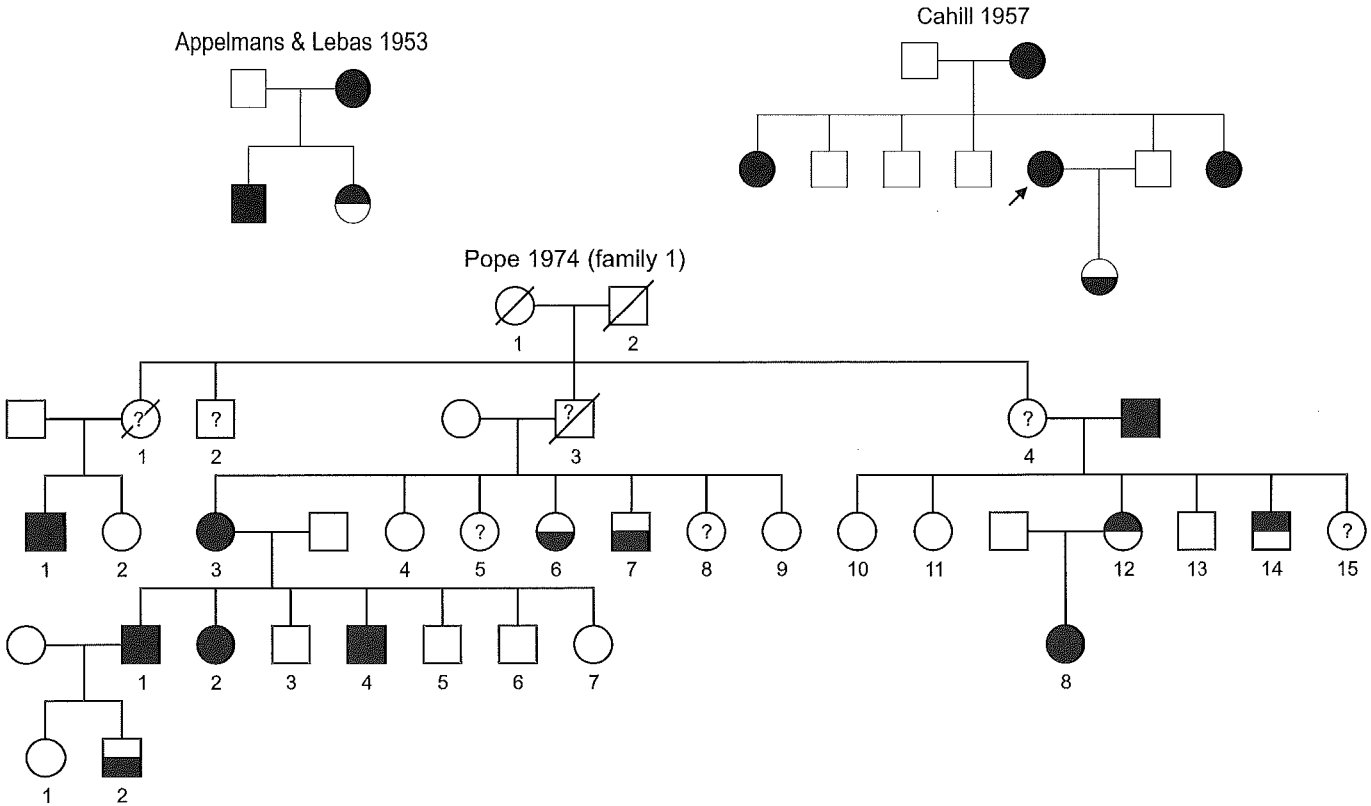


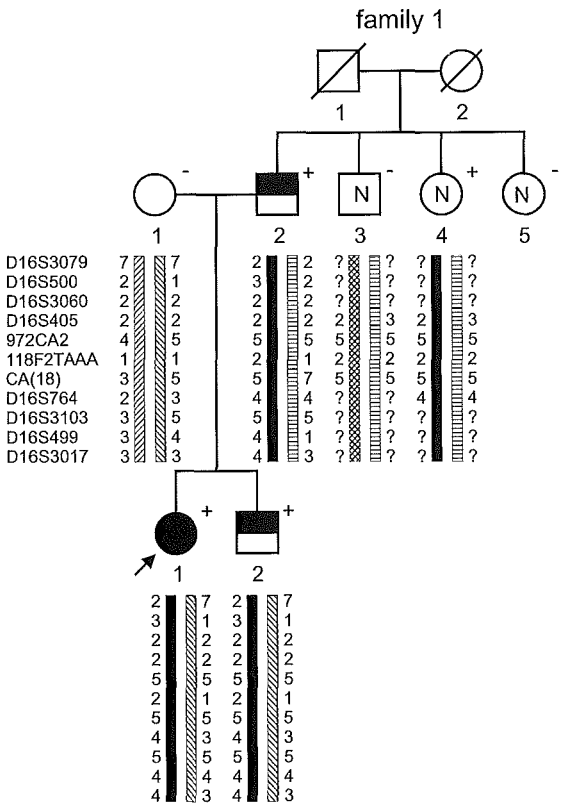
Figure 1 Pedigrees from the literature, in which definite PXE was present in two or more generations, adapted to our criteria. We only included the relevant family members. Square, male; circle, female; square/ circle with slash, deceased; deceased; black upper half, ophthalmologic PXE signs; black lower half, dermatologic PXE signs;? Said to be affected, no further data; arrow points to proband.

Table I Literature on PXE in two or more generations. Only relevant family members have been included

Authors	Patient	age (y)	Eyes	Skin	Other abnormalities
Weve [1934]	male	54	AS	'very mildly affected'	
	son		normal	'obvious PXE'	
Denti [1938]	female proband	37	AS, bleeding	skin abn. on neck and elbows	
	mother			similar abn.	
Kat and Prick [1940]	female proband	24	AS	characteristic of PXE, pos. biopsy	goitre
	father			'indication of PXE on neck and elbows'	
Osbourn and Olivo [1951]	female proband	29	AS	characteristic of PXE, pos. biopsy	
	daughter	3	normal	yellowish, wrinkling on neck	
Coffman and Sommers [1959]	female proband	68	?	'biopsy typical of PXE'	valvular heart disease
	3 daughters		no signs of PXE	no signs of PXE, pos. biopsy	variable cardiac abn.
	2 sons		no signs of PXE	normal, pos. biopsy	one: hypertension
Capusan et al. [1960]	female proband	24	AS	characteristic of PXE, pos. biopsy	Albers-Schönberg disease
	mother			similar skin abn.	
Gills and Paton [1965]	male proband	14	AS, bleeding, peau d'orange	characteristic of PXE	
	mother		peau d'orange	normal	
Hull and Aaberg [1974]	female	63	AS	'compatible with PXE'	
	sister	60	AS, pigment clumping	'compatible with PXE'	
	brother	55	AS	'PXE-biopsy proven'	
	-his daughter	25	AS	Normal	
	-his daughter	16	AS	Normal	
	sister	52	AS, peau d'orange	coarse furrows in neck	
	-her son	14	L: atrophic choroidal crescent temporal to disk	Normal	
Cunningham et al. [1980]	female proband		AS, MD	characteristic biopsy	
	father		MD		
	daughter		peau d'orange		
Bao et al. [1991]	female proband	25	AS, peau d'orange	yellow papules and plaques	

	mother	54	peau d'orange	Papules	
Hausser and Anton-Lamprecht [1991]	female proband	47	visual impairment	'typical lesions', pos. biopsy	
	mother			'typical symptoms'	hypertension, vascular abn.
	son	18	normal	suspicious in knee flexures, pos. biopsy	
	twin daughters	17	normal	normal, pos. Biopsy	
Katagiri et al. [1991] -case 12	female proband	21	AS, peau d'orange	peau d'orange, cutis laxa	
	mother			'similar'	
-case 7	male proband	48	AS, bleeding, peau d'orange	peau d'orange, cutis laxa	AP, interm. claudication
	M, MM, MMM			'alleged to be similar'	
Ekim et al. [1998]	female proband	11	AS, peau d'orange	papules, pos. biopsy	cardiovascular abn.
	mother	35	peau d'orange		increased echogenicity of kidneys and pancreas
Appelmans and Lebas [1953]*	female proband	50	AS, bleeding, MD	characteristic of PXE	hypertension
	son	28	AS, yellowish retinal lesions	many papules high on back	
	daughter	25	AS, yellowish retinal lesions	Normal	
Cahill [1957]*	female proband	39	AS, MD	characteristic of PXE	cardiovascular abn.
	mother	72	choroidoretinitis, blind, AS?	characteristic of PXE	
	daughter	18	normal fundus	early skin changes	
Pope [1974] -family 2	female proband	42	AS	faint rash on neck, flexures	
	mother	69	mild 'salmon spotting'	'macular PXE'	
	daughter	19	blue sclerae, mottling, prominent choroidal vessels	faint rash neck, hyperextensible	hypermobile joints, pectus excavatum, high palate
	son	18	mottling, prominent choroidal vessels	'macular PXE', hyperextensible	high palate

abn., abnormalities; AP, angina pectoris; AS, angioid streaks; GI, gastrointestinal; interm., intermittent; L, left; M, mother; MD, macular degeneration; MM, mother of M; MMM, mother of MM; pos., positive; 59†,deceased at age 59; definite PXE in two or more generations.



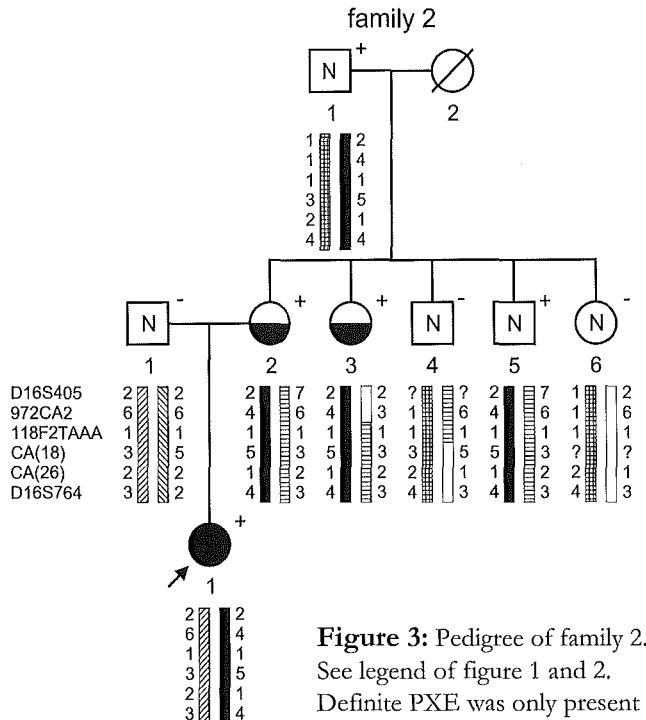


Figure 3: Pedigree of family 2. See legend of figure 1 and 2. Definite PXE was only present in III-1.

Figure 2: Pedigree of family 1. Bars represent haplotypes of microsatellite markers flanking the ABCC6 gene. The gene is located between the markers 118F2TAA and D16S764. See figure 1 for symbol definition; -, mutation absent; +, mutation present; N, unaffected. Definite PXE was only present in III-1.

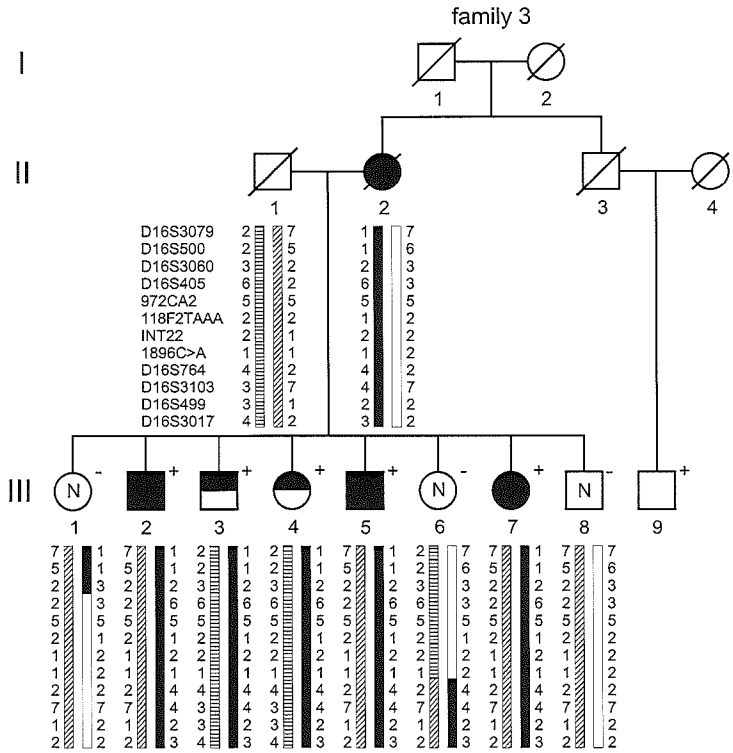


Figure 4: Pedigree of family 3, with definite PXE in two generations. The haplotypes in generation II were reconstructed.

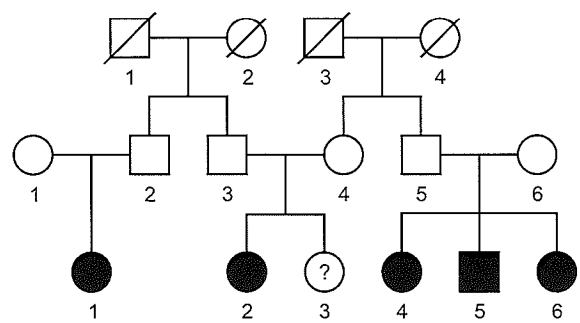


Figure 5: Pedigree of a family with PXE in the three nuclear families. No consanguinity has been found between the presents in the second generation. III-6 had two different mutations, which were not found in III-1. This pedigree illustrates that (carrier) frequency of PXE could be much higher than previously assumed.

Family 1. The pedigree with clinical and DNA data is presented in figure 2. The female proband (III-1) was first seen by an ophthalmologist at age 27, because of perceived loss of visual acuity. Upon examination visual acuity was normal, but fundoscopy of both eyes did reveal angioid streaks and peau d'orange. Skin abnormalities on the neck had been noticed since age 4 years. Recent examination by a dermatologist revealed yellowish papules and plaques on the neck, the axillae and antecubital fossae. Histopathologic analysis of a skin biopsy revealed changes typical for PXE. The cardiologist did not find signs of cardiovascular disease. SSCP, direct sequencing and extensive southern analysis showed a single 4-basepair insertion in exon 30 (4220insAGAA) in a only one *ABCC6* allele.The other allele did not reveal any non-synonymous sequence changes. The paternal grandfather (I-1) was said to have had a thickened skin of the neck. He died suddenly at age 79 due to a cerebrovascular accident. The paternal grandmother (I-2) had a cerebrovascular accident at age 81. The mother (II-1) had a normal fundus on ophthalmologic examination and no skin abnormalities. No *ABCC6* mutation was found in her DNA by SSCP and sequencing. The father (II-2), aged 51, had normal visual acuity, peau d'orange, angioid streaks, some yellowish papules in the neck (too few to be typical for PXE), and a negative skin biopsy. The cardiologist did not find any abnormalities. The father did have the same mutation as his daughter in one allele. An uncle and two aunts (II-3, II-4, II-5) did not have any ophthalmologic or dermatologic abnormalities on clinical examination. Only one of them (II-4, aged 49) underwent a skin biopsy, that was normal. She had the *ABCC6* mutation in one allele, her brother (II-3) and sister (II-5) did not. Fundoscopy of the 24-year-old brother (III-2) of the proband revealed peau d'orange and angioid streaks. Three years later he experienced loss of vision, caused by retinal hemorrhage due to a slap on his eye. He did not have evident skin abnormalities, had a normal skin biopsy and no signs of cardiovascular disease. DNA studies showed the same mutation in one allele and, for both alleles, the same haplotypes as in his sister. In summary, the proband had definite PXE, while her brother and father only had ophthalmologic signs. All three, and a healthy aunt, were heterozygous for the same *ABCC6* mutation.

Family 2. The female proband of this family (III-1, figure 3) had progressive skin abnormalities in the neck since age 8 years. The dermatologist saw yellowish papules and plaques, mainly on the neck and less pronounced on the axillae and periumbilical area. Histopathologic study of a skin biopsy showed abnormalities

characteristic of PXE. Ophthalmologic examination at age 14 showed peau d'orange and one comet-like streak. Extensive screening of the *ABCC6* gene, including SSCP, genomic sequencing of all exons, and southern analysis, revealed a single R1141X mutation in only one allele. The other ABCC6 allele is wildtype.

The maternal grandfather (I-1) was said to have no skin abnormalities. Ophthalmologic examination did not reveal any signs of PXE. However, he did have the R1141X mutation. The father (II-1) was normal. Examination of the mother (II-2) revealed some skin papules, mainly at the right cubital fossa. Histopathologic study of a skin biopsy showed abnormalities characteristic of PXE. She did not have ophthalmologic abnormalities. She also had the R1141X mutation in a single allele by the sequencing of the ABCC6 gene. An aunt (II-3) had some yellowish papules on the neck and the cubital fossae. Her skin biopsy showed mild abnormalities in accordance with PXE. At fundoscopy no signs of PXE were noticed. She also was heterozygous for the R1141X mutation. Two uncles and the youngest aunt (II-4, II-5, II-6) all had some yellowish papules at the cubital fossae, not characteristic of PXE. Their skin biopsies and ophthalmologic examinations did not show any abnormalities. One of the uncles had the R1141X mutation. In summary, only the proband had definite PXE. Her mother and aunt only had minimal skin abnormalities. All three, a healthy uncle and the grandfather had the same mutation in one allele.

Family 3. The mother (II-2 in figure 4) had noticed acute vision loss of the left eye at age 62. On fundoscopy a peripapillary hemorrhage was seen in addition to disciform macular degeneration and angioid streaks. Dermatologic examination showed skin lesions typical for PXE. Histopathology of a skin biopsy, taken in 1973, revealed thickening, fragmentation and clumping of elastic fibres. No DNA was taken from her before her death. Her haplotype was reconstructed. However, the presence of an R1459C mutation in the *ABCC6* gene of a nephew (III-9) indicated that she transmitted this mutation to her affected children (figure 4). She had eight children. Two daughters (III-1, III-6) and a son (III-8) had no signs of PXE on examination by both the ophthalmologist and dermatologist. They did not have the R1459C mutation. The eldest son (III-2) noticed visual deterioration at age 48. He had disciform macular degeneration and skin abnormalities characteristic of PXE. Extensive screening of the *ABCC6* gene revealed an R1459C mutation in one allele only. Direct sequencing of the entire cDNA, derived from both alleles, showed one mutated (R1459C) and one wild type *ABCC6* transcript

(not shown).Son III-3 was examined at age 61. He had normal visual acuity and angioid streaks. The dermatologist saw some yellowish papules on the neck and axillae, not enough to be characteristic of PXE. Histopathology of a skin biopsy showed doubtful increase of elastic fibres, not conclusive for PXE. He did have the R1459C mutation. Daughter III-4 was examined at age 59. Visual acuity was normal and on fundoscopy peripapillary atrophy was noted. Fluorescein angiography showed peau d'orange and angioid streaks in both eyes. Dermatologic examination, including a skin biopsy, did not reveal any signs of PXE. She had the R1459C mutation in one allele. Son III-5 had visual deterioration at age 55, caused by retinal detachment of the right eye. He had angioid streaks in both eyes and pigmentary changes in the left macula. One year later he had a hemorrhage in this eye. The dermatologist saw yellowish papules in the subclavicular/presternal area. Histopathologic study of a skin biopsy showed some clumping of elastic fibres. He also had the R1459C mutation in one allele. The youngest daughter (III-7) noticed visual deterioration at age 48. She had angioid streaks and choroidal neovascular membranes in both eyes, for which she had laser therapy. The dermatologist found yellowish papules on the neck, the axillae and antecubital fossae. Histopathologic study of a skin biopsy showed clumping and fragmentation of elastic fibres. DNA studies showed the R1459C mutation in one allele.

In summary, in this family definite PXE occurred in two generations. *ABCC6* transcript analysis showed the presence of one mutated (R1459C) and one wild type allele in all affected family members.

Discussion

Literature on autosomal dominant PXE families

The unusually high frequency of AD inheritance (53%), found by Pope, can be explained by the fact that Pope erroneously allocated sporadic patients to an AD type, only on grounds of their clinical pattern. Pope described two AD families more extensively (Table 1). The pedigree of his family 1 is presented in figure 1, in which we only show the data that were available from the text. Persons with a question mark were said to be affected, but no further data were given. In most patients the only ophthalmic sign mentioned was '(choroido)retinopathy'. If we assume that this consisted of signs of PXE, AD PXE with reduced penetrance is

most likely. Signs of PXE were present in four generations with father-to-son inheritance. Pseudodominance is unlikely, because definite PXE was present in three nuclear families (offspring of a brother and two sisters), although it is not clear whether consanguinity could have played a role. In his family 2 (Table 1) we can not be sure about the diagnosis PXE, partly due to lack of detailed data. The hyperextensible skin and hypermobile joints in this family could also point to Ehlers-Danlos syndrome, that is also associated with angioid streaks.

From our literature search, we selected two more reports of probably dominant PXE families (Figure 1). In these families, described by Appelmans & Lebas[1] and Cahill,[7] respectively, definite PXE was present in one generation, probable PXE in the second generation, and, in the latter, only one diagnostic criterion in the third generation. Appelmans & Lebas did not mention the possibility of consanguinity. In the family reported by Cahill no history of consanguinity was said to be obtainable. Pseudodominance can not be excluded in these two small families. Pseudodominance has been reported before[36] and becomes more likely if the carrier frequency of the disease is high. *ABCC6* mutation analysis of a control population of 1057 persons in our lab yielded 8 carriers of the R1141X mutation.[41] This mutation appears to make up one third of all *ABCC6* mutations in our PXE population, so that PXE carrier frequency could be as high as 2.4%. This is much higher than expected on the basis of the earlier mentioned prevalence of 1 in 100.000, by which the carrier frequency would be 0,6%. This is also supported by the fact that we know an AR family in which an aunt and her niece had PXE, and an AR family, in which at least five cousins in three nuclear families (see figure 5 for the latter) were affected, without indications for consanguinity of the parents. Consequently, pseudodominance could be a common phenomenon in PXE.

Our family studies

In family 1 PXE seemed to be present in two generations (figure 2). While the index patient had definite PXE, her brother and father only had ophthalmologic signs of PXE, the brother more severe than the father. The mutation in this family was a 4-basepair insertion in exon 30, which has not been found yet in other patients. One possibility is that this mutation can cause AD PXE. In that case, penetrance would be reduced, because a healthy female (II-4, aged 49) in the second generation had the mutation. Similarly, one of the grandparents (first

generation) probably had the mutation. However, the grandfather (I-1) only was known to have had thickened skin of the neck and a cerebrovascular accident at age 79, the grandmother a cerebrovascular accident at age 81. Obviously, this is too little to make a diagnosis of PXE. Another possibility is AR inheritance. The index patient and her brother (III-1 and III-2) could have had a (yet undetected) mutation in their second allele, which they shared. Their father could have had a milder expression due to the heterozygous state. Mild skin and ophthalmologic abnormalities in putative heterozygote carriers of PXE have been reported.[2,5,37]

In our second family, a 14-year old girl (III-1) had definite PXE. Her mother and aunt only had mild skin abnormalities, that could be expression of a heterozygous state. The R1141X mutation was found in one allele of these three individuals and in a healthy uncle. This mutation has been found in 30% of alleles of PXE patients, heterozygous, combined with other mutations (compound heterozygous), as well as homozygous. We did not find additional possible AD families with this mutation. Expression studies in our lab suggested that the R1141X mutation leads to absence of protein by nonsense-mediated RNA decay.[18] In that case AR inheritance is most likely. The patients with definite PXE, in whom one R1141X mutation was found, could have a second, as yet unknown, mutation.

In our third family, all available clinical, genealogical, genetic, molecular and allelic expression data pointed towards AD inheritance, although variable expression within the pedigree existed. It is remarkable that the three most seriously affected sibs (III-2, III-5 and III-7) had exactly the same haplotypes, including the disease-associated haplotype. In contrast, the two sibs with a milder phenotype (III-3 and III-4) shared another second allele. Theoretically, this could point to pseudodominance and AR inheritance with partial expression in heterozygotes. In that case, the father should have had one and the mother two mutations. Given the molecular data and segregation of markers in the pedigree this is very unlikely. First, we would have missed two different *ABCC6* mutations, one in the DNA of the mother and one in the DNA of the father. Second, if both *ABCC6* haplotypes of mother carry a mutation, all sibs would obviously have inherited at least one mutation. If one of the paternal haplotypes would also carry an *ABCC6* mutation it is evident, given the segregation of markers in the pedigree, that either III-1, III-2, III-5, III-6, III-7, III-8 or, alternatively, III-3 and III-4, would have inherited a paternal *ABCC6* mutation. Given the healthy, non-PXE, phenotype of III-1, III-6 and III-8, it is highly unlikely that they have two (one

maternal, one paternal) *ABCC6* mutations. Alternatively, III-3 and III-4 could have two *ABCC6* mutations, and the other sibs from the second generation only one. This is also unlikely, since III-3 and III-4 presented with a milder phenotype (angioid streaks only, no skin lesions) than the other affected sons and daughter. Taken all data together, AR inheritance with pseudodominance in this pedigree can be virtually excluded.

Does AD PXE exist?

In the literature we found only three families in which AD PXE seemed likely. In two of these there could be pseudo-dominant inheritance, in the third one this was unlikely. In other families, there was no definite PXE in two or more generations, but only part of the phenotype appeared to be present in a second generation. Partial expression could be due to the heterozygous state of an AR inherited disease, which is also possible in our families 1 and 2. Recently, heterozygosity for the R1141X mutation was found to be associated with increased risk of cardiovascular disease.[41] Expression in heterozygotes has also been described in other diseases caused by mutations in ATP-binding cassette (ABC) transporter genes. Mutations in both ABC1 alleles cause Tangier disease, while heterozygous mutations have been found in families with AD HDL-cholesterol deficiency, which is a much milder phenotype [Marcil et al., 1999]. Subjects heterozygous for mutations in CFTR (ABCC7, the cystic fibrosis gene) may have an increased risk for disseminated bronchiectasis and sarcoidosis [Bombieri et al., 1998]. Heterozygous mutations in ABCA4, the gene for AR Stargardt disease, may increase the risk for age-related macular degeneration.[39] Comparable with this, an ABCC6 mutation in heterozygote carriers usually does not result in pathology, depending on other genetic or environmental factors. Obviously, the clinical classification of the heterozygotes determines whether or not the mode of inheritance is AD or AR. Given the earlier mentioned uncertainties in clinical classification and pathogenesis of PXE it should be kept in mind that part of the problem in determining the inheritance mode in our families still may be created by misclassification. For accurate genetic counselling we will have to know in due time the expression of the specific alleles in homozygous, compound heterozygous and heterozygous states, as well as the possible influence of other loci. Our family 3 shows that R1459C might be a mutation that can cause PXE in the heterozygous state.

In summary, we conclude that AD inheritance in PXE may exist, but that it is much rarer than previously assumed (1/59 (1,7%) of our population), and probably has low penetrance. More detailed clinical and molecular studies of families with (features of) PXE in two or more generations should shed further light on this issue. At this moment it seems that offspring of patients with PXE does have a slightly increased risk of symptoms of PXE, but full-blown PXE in two or more generations is very rare.

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Chapter 7

MRP6 (ABCC6) Detection in Normal Human Tissues and Tumors

MRP6 (ABCC6) is a member of the subfamily of the multidrug resistance protein (MRPs, reviewed by Borst et al, 2000), but its putative role in multidrug resistance (MDR, reviewed by Moscow et al, 1997) is still under investigation. Closely related proteins such as MDR1 P-glycoprotein (P-gp, ABCB1, reviewed by Ambudkar et al, 1999), breast cancer resistance protein (BCRP, ABCG2; Doyle et al, 1998), and MRP1, -2, and –3 (ABCC1-3) are established MDR transporters. The exact range of substrates for MRP6 has not been determined, but a preliminary report suggested that MRP6 may be involved in the transport of certain anticancer agents, including anthracyclines and epipodophyllotoxins (M.G. Belinsky et al, Proceedings of the AACR, abstract 1510, 2001). Recently it was found that mutations in the MRP6 gene cause pseudoxanthoma elasticum (PXE), an inheritable disorder of the connective tissue involving impaired visual acuity, skin lesions, and cardiovascular complications (Bergen et al, 2000). The expression of MRP6 in normal human tissues has only been studied at the mRNA level. High MRP6 mRNA levels were reported in liver and kidney, whereas low expression was found in a range of other tissues, including lung, intestines, retina, skin, and vessel walls (Bergen et al, 2000; Kool et al, 1999).

To study MRP6 at the protein level, three rat Mabs (M_6II-7,M_6II-21, and M_6II-31) were generated from rats immunized with a fusion protein containing amino acids 746 to 946 of human MRP6 (FP M_6II), according to described methods (Scheffer et al, 2000). Reactivity of these Mabs to full length MRP6 protein was shown in Western blots with fractions of MRP6-overexpressing HEK 293 cells. All Mabs reacted with the approximately Mr 180,000 MRP6 protein, that migrated slightly faster than the related MRP2 protein (ABCC2), as detected with the M_2III-6 Mab (Scheffer et al, 2000) in a control cell line (Fig. 1). Isotype specific secondary antibodies (Nordic, Tiburg, The Netherlands) showed that M_6II-7 and M_6II-31were both of IgG2a subclass, whereas Mab M_6II-21was of IgG1 subclass. Lack of cross reactivity of M_6II-7, M_6II-21, and M_6II-31 with MDR1 P-gp or MRP1, -2, -3, -4, and –5 family members, was concluded from staining results from cytospin preparations of several cell lines and Western blot experiments with protein fractions of these cell lines (Table 1). In line with normal tissue distribution studies a t the mRNA level, considerable MRP6 staining in frozen sections of normal human tissues appeared to be restricted to kidney and liver. In the other tissues examined, including lung, brain, bladder, tonsil, spleen, heart, colon, adrenal land, gall bladder, skeletal muscle, testis, ovary, placenta, parotis, esophagus,

stomach, and pancreas, no MRP6 staining was observed. Surprisingly, also in sections of human skin and retina, no MRP6 staining could be observed, despite the fact that mutations in MRP6 were implicated in the connective tissue disorder in PXE. Ongoing studies are investigating the direct or indirect role of (mutated) MRP6 in the eye and in skin lesions of PXE patients.

In kidney, MRP6 was located at the basolateral membranes of the proximal tubules (Fig. 2a). The staining pattern paralleled the staining observed with the anti-MRP2 Mab M_2III-6, but, as previously reported (Scheffer et al, 2000), the latter Mab rather stained the apical membranes (Fig. 2c). control staining with anti-Tamm-horsfall polyclonal antiserum showed that this antigen does not co-localize with MRP6 (Fig. 2d). The Tamm-horsfall protein is localized in the early distal convoluted tubules and the thick ascending loops of Henle.

In liver, the anti-MRP6 Mabs strongly stained the membranes of the hapatocytes (Fig. 3a). In contrast to data obtained with a polyclonal antiserum in rats (Madon et al, 2000), the staining appeared to be located exclusively at the basolateral membranes, leaving, at closer look, especially the cross-sections of the canalicular membranes as particularly unstained region in a marked pattern. A control staining for MRP2 (known to be present at the canalicular membranes [Scheffer et al, 2000]) is shown in Figure 3c. To further investigate the presence or absence of MRP6 at the hepatocanalicular membranes, we performed a double staining fro MRP6 and MRP2 using secondary regents labeled with different fluorochromes. The proteins appeared to be present mutually exclusive at the basolateral and canalicular membranes, respectively, as shown by the absence of mixed fluorochrome color (Fig.3d)

Examination of the reactivity of the anti-MRP6 Mabs on formalin fixed, paraffin ambedded sections of human liver, using the citrate method as antigen retrieval, showed that the M6II-7 and M6II-21 Mab were unreactive on this type of material, whereas the M6II-31 Mab was reactive, albeit less than observed in frozen sections.

In frozen sections of a small panel of human tumor samples from tissue of different histogenetic origins (including tumors of the intestine, stomach, testis, prostate, lung, pancreas, bladder, adrenal gland, cervix, neurologic tissue, mamma, ovary, kidney, and melanoma, n = 34), no clear MRP6 levels could be detected, including that the contribution of MRP6 to MDR in tumors may be rather limited.

The MRP6 specific rat Mabs should become valuable tools in further studying the substrate specificity and possible contribution of MRP6 to MDR and they should facilitate in-depth studies into the nature of the disease in PXE patients.

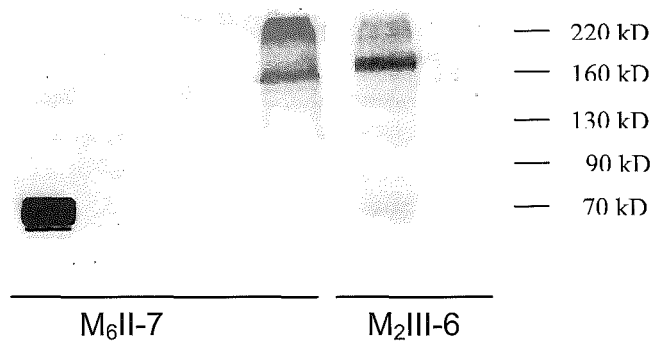

Figure 1 Detection of the approximately 65 kD MRP6-MBP fusion protein and full length MRP6 from protein preparations of MRP6-transfected HEK 293 cells, using anti-MRP6 Mab M6II-7. The approximately 180,000 kD MRP6 migrates slightly faster than MRP2, as shown by control lanes stained for MRP2, using M2III-6 and MRP2-transfected cells. Total cell lysates were made as described (Scheffer et al,2000). 10 - 40 μg of cell lysates or fusion proteins were fractionated on a 7% polyacrylamide slab gel and transferred onto a nitrocellulose membrane by electroblotting. After blocking, the membrane was incubated for 2 h with primary antibody in the appropriate dilution. Horse radish peroxidase (HRP)-labeled-anti-rat serum or –mouse (1:1000, Dako) was used as a secondary antibody. Enhanced chemoluminescence (Amersham, U.K.) was used to detect Mab binding.

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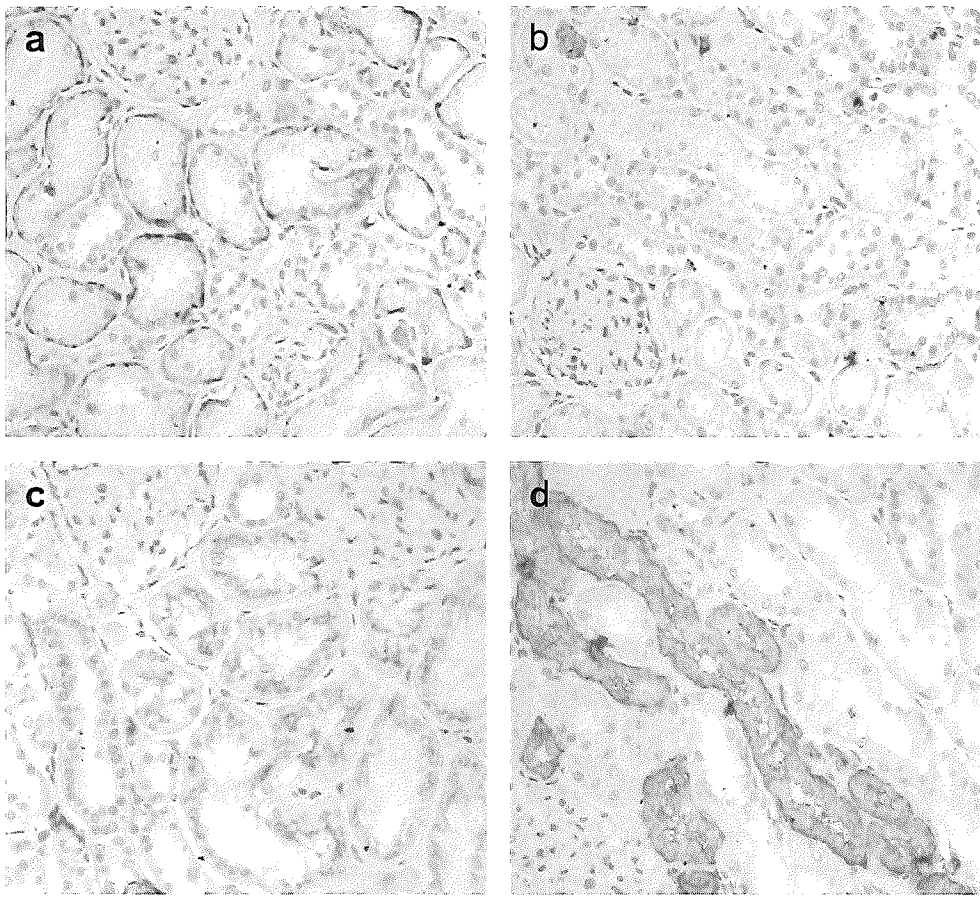

Figure 2

Immunohistochemical staining of normal human kidney, using HRP-labeled rabbit-anti-rat serum, fluorescein isothiocyanate (FITC)-labeled tyramine, HRP-labeled rabbit F(ab')2 - anti-FITC and aminoethyl carbazole as the chromogen. M6II-31 shows the presence of MRP6 in the basolateral membranes of the proximal tubuli (a). Control stainings are with negative control antibody (b), anti-MRP2 mouse Mab M2III-6 (c) and anti-Tamm-Horsfall rabbit polyclonal antiserum (d). M2III-6 stains the apical membranes of the proximal tubules. Rabbit polyclonal antisera against kidney Tamm-Horsfall protein was a kind gift of Dr. R.A.M.H. van Aubel, University of Nijmegen, the Netherlands. The Tamm-Horsfall protein is localized in the early distal convoluted tubules and the thick ascending loops of Henle.

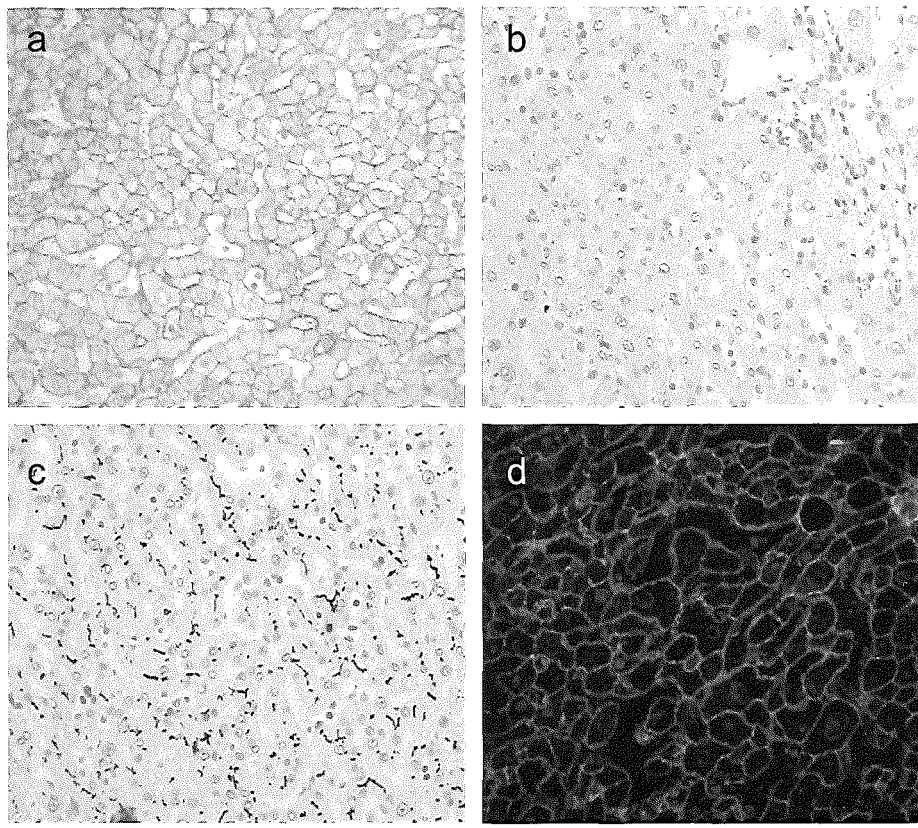

Figure 3

Immunohistochemical staining of normal human liver, using HRP-labeled rabbit-anti-rat serum, FITC-labeled tyramine, HRP-labeled rabbit F(ab')2 -anti-FITC and aminoethyl carbazole as the chromogen. M6II-31 shows the presence of MRP6 in the basolateral membranes of the hepatocytes (a). Control stainings are with negative control antibody (b) and anti-MRP2 Mab M2III-6 (c). M2III-6 stains the canalicular membranes of the hepatocytes. In (d) a fluorescent double staining for MRP6 and MRP2 is shown, using rat anti-MRP6 Mab M6II-31, HRP-labeled rabbit-anti-rat and tyramine-FITC (green), followed by staining with mouse anti-MRP2 Mab M2III-6, biotin-labeled rabbit-anti-mouse and Cy3-labeled steptavidin (red). Nuclei were counter stained with 4',6-diamidino-2-phenylindole (DAPI; blue). MRP6 and MRP2 are present mutually exclusive at the basolateral- and canalicular membranes, respectively; no mixed fluorochrome color is observed.

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Table 1 Reactivity of monoclonal antibodies in cell lines

			Mab[d]								
Cell line	origin	Transporter	JSB-1	MRPr1	M_2III-6	M_3II-9	M_4II-8	M_5I-1	M_6II-7	M_6II-21	M_6II-31
SW1573 [a]	lung		-	+/-	-	-	-	-	-	-	-
SW1573/2R160 [a]		MDR1 P-gp	+++	+	-	-	-	-	-	-	-
GLC4 [a]	lung		-	+/-	-	-	-	-	-	-	-
GLC4/ADR [a]		MRP1	-	+++	-	-	-	-	-	-	-
HL60 [a]	leukemia		-	-	-	-	-	-	-	-	-
HL60/ADR [a]		MRP1	-	++	-	-	-	-	-	-	-
2008 [a]	ovarian		-	+	-	-	-	-	-	-	-
2008/MRP1 [a]		MRP1	-	+++	-	-	-	-	-	-	-
2008/MRP2 [a]		MRP2	-	+	+++	-	-	-	-	-	-
2008/MRP3 [a]		MRP3	-	+	-	+++	-	-	-	-	-
Sf9/MRP4 [b]	insect	MRP4	-	-	-	-	+++	-	-	-	-
HEK293/MRP5 [a]	kidney	MRP5	-	-	-	-	-	+++	-	-	-
HEK293/MRP6 [c]	kidney	MRP6	nd	nd	nd	nd	nd	nd	+++	+++	++(+)

-, no reactivity; +/-, very weak reactivity; +, weak reactivity; ++, moderate reactivity; +++, strong reactivity; nd, not determined;

[a] (Scheffer et al, 2000).

[b] Spodoptera frugiperda Sf9 insect cells, transiently transfected with full length MRP4 cDNA were a kind gift of Dr. R.A.M.H. van Aubel, University of Nijmegen, the Netherlands.

[c] protein fractions of HEK293 cells transfected with full length MRP6 cDNA were a kind gift of Dr. J. König, German Cancer Research Center, Heidelberg, Germany. [d] (Scheffer et al, 2000) and unpublished

Chapter 8

Conclusion and General Discussion

To gain further insight into the molecular pathology of pseudoxanthoma elasticum (PXE), we investigated the function of PXE disease gene, *ABCC6*, through clinical examination, and with molecular genetic and immunohistochemical approaches. In this chapter, the main outcomes of our studies and their implications for the mechanisms underlying the disease will be addressed. Furthermore, prospects for further studies, partly based on unpublished data, will be discussed.

ABCC6 mutations and its functional consequences in PXE

The mutation analyses of the *ABCC6* gene in PXE patients was described in chapters 2 and 4. We have investigated 76 PXE families and patients. In total, we identified 20 different sequence changes. Among these, 14 were new variations. Putative disease-causing mutations were found on 89 out of 152 *ABCC6* alleles (58.6%). We identified 61 patients (80.3%) who carried at least one affected allele. This detection rate in our group was comparable to another report in a European cohort of 61 cases.[18] However, the following possibilities of missing a number of mutations should be kept in mind. Nucleotide changes deep into introns may escape screening based on PCR amplification of the coding regions and exon-intron boundaries. Also false negative results could be obtained by Southern blot analysis due to the presence of *ABCC6* pseudogene sequences of the first nine exons. Finally, a number of patients with only one mutant allele may express mild or full PXE due to other genetic and/or environmental factors.

The whole spectrum of types of mutations was found in the *ABCC6* gene. Nonsense mutations accounted for 41.6 percent of the PXE-associated mutations. Furthermore, frameshift (23.6 percent), out of frame deletion (19.2 percent), missense (11.2 percent), splice (2.2 percent), and entire *ABCC6* gene deletions (2.2 percent) constituted the remaining mutations. The most frequent mutations in 152 ABCC6 alleles studied were a nonsense mutation (R1141X, 23.0%) and deletions (del exons 23-29, 11.2%; 3775del T, 7.2%; 4182delG; 4.0%) described in Table 2, Chapter 4. These mutations are expected to cause either a shorter or dysfunctional mRNA or an absent or dysfunctional protein.

Family studies and *ABCC6* mutation data suggested that in the majority of cases the PXE phenotype might be attributed to loss of functional ABCC6 protein. To further analyse this hypothesis, a single nonsense mutation, R1141X, was studied in more detail in chapter 3.

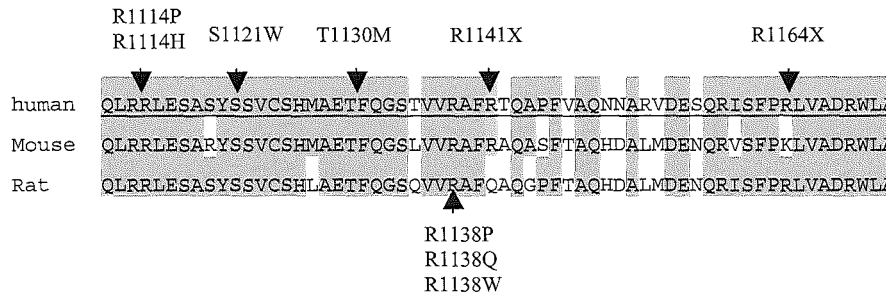
The distribution pattern of *ABCC6* mutations is unequal as shown in chapter 2. The majority of mutations occurred toward the carboxy-terminal end. Almost all mutations were located in the cytoplasmic domains of the protein and clustered in NBF1, NBF2, and the eighth cytoplasmic loop. This distribution pattern reflects the functional significance of evolutionary conserved ATP-binding domains, nucleotide binding folds (NBFs).[11] Functional studies of a number of ABC proteins indicated that these two NBFs are critical for ATPase and for ATP driven transport function.[7,27] Within the *ABCC* gene family, ABCC1 is highly similar in amino acid sequence (54.8%) to ABCC6.[16] Previous mutation analysis of ABCC1 indicated that marked reduction in transport function is caused by missense mutations in NBF1 and NBF2.[10] Recently, Ilias et al investigated three missense mutations (V1298F, G1302R, G1321S) within NBF2 in the *ABCC6* gene.[13] They found that the *ABCC6* gene with those mutations yielded proteins of similar size and about the same amount as the wild type, but with complete loss of transport function in Sf9 insect cells. Although it is not clear whether those results are representative for all *ABCC6* missense mutations, this finding provides a clue that PXE is caused by loss of ABCC6 transport function.

Another cluster of mutations, including two nonsense and seven missense mutations, occurred in the eighth cytoplasmic loop, as indicated in figure 1 in chapter 2. Multiple different mutations in the same loop suggest that this particular region may be essential for the function of ABCC6. Highly conserved sequences in the eighth loop of human ABCC6, mouse Abcc6, and rat Abcc6 support this hypothesis (Fig 1). However, the precise function of this region remains to be elucidated.

Characterization of the most common mutation in ABCC6/PXE: R1141X

To increase our understanding of the molecular pathology of PXE, we characterized the most common mutation, R1141X, in more detail in chapter 3. The R1141X mutation accounted for 39.1% of the mutant alleles of *ABCC6* in PXE patients in the Netherlands. Haplotype analysis was performed with markers spanning and flanking the *ABCC6* locus in 16 patients with PXE with the R1141X mutation. The results revealed that R1141X co-segregated with a conserved haplotype. This indicated that the mutation is very old and that a founder origin of R1141X exists in the Netherlands. The R1141X mutation was present in homozygous (3/16), compound heterozygous (6/16), or heterozygous (7/16) form.

Figure1 Alignmentsof 8th cytoplasmicdomain human ABCC6, mouse Abcc6, and Rat Abcc6, showing the evolutionary conserved nature of the region



These patients were either sporadic cases or were members of families in which autosomal recessive (AR) inheritance was the most likely segregation pattern.

The identification of a founder mutation in the Netherlands greatly simplified DNA diagnostic services. In a number of patients with the R1141X mutation no mutation was found on the second allele. It is likely that a number of potential mutations in the second allele escaped attention by current mutation analyses. Alternatively, whether a single R1141X allele is enough to express a mild PXE phenotype remains to be investigated. To characterize the functional consequences of R1141X, we studied both mRNA transcripts and the ABCC6 protein. Theoretically, the R1141X mutation may lead to either a null allele produced through nonsense mediated decay of *ABCC6* mRNA or an ABCC6 protein lacking 362 amino acids at the carboxy-terminal domain including NBF2. Steady-state mRNA in cultured skin fibroblasts from a patient homozygous for R1141X was investigated and no detectable *ABCC6* mRNA was found. Fibroblasts from patients heterozygous for the R1141X mutation appeared to have a reduced level of *ABCC6* mRNA compared with healthy control subjects. Subsequent restriction enzyme digestion showed transcripts from a wild type *ABCC6* sequence in the R1141X heterozygous persons only. Predominant expression of the wild type allele was also observed in mononuclear blood cells, in which only 5% of the mRNA was from a mutant allele. In a parallel study, with mononuclear blood cells from subjects heterozygous for the R1459C missense mutation, random expression of wild type and mutant alleles was identified. The R1141X transcripts were highly unstable and probably degraded by nonsense-mediated decay (NMD).[9] The

expression differences between fibroblasts and blood might be caused by tissue specificity of the NMD process like for example collagen X gene in Schmid metaphyseal chondrodysplasia.[4] In the latter, a nonsense mutation for collagen X gene led to complete NMD in cartilage cells but not in non-cartilage cells.

In parallel experiments, immunocytochemical staining with monoclonal antibodies against ABCC6 was performed on cultured dermal fibroblasts from a subject homozygote for R1141X. The result showed no evidence of the presence of a (truncated) protein. In conclusion, our results provide evidence for the hypothesis that R1141X results in a functional null allele. We assume that a complete loss of ABCC6 function in patients for homozygous R1141X mutation is the cause of PXE.

Efficient strategy for the molecular diagnosis of PXE

In chapter 4, we designed a new and efficient molecular screening strategy for *ABCC6*. This strategy condensed our initial screening protocol to four phases. The protocol is based on PCR amplification combined with restriction digestion and denaturing high performance liquid chromatography (DHPLC) detection supplemented with Southern blot analysis when necessary. The most frequent mutations were analysed by using simple and efficient approaches. Using this strategy, 78.7% of all *known* mutant alleles could be screened for each patient within a single week by one technician. With a mutation rate of approximately of 80.3% (mutations per patient), our strategy is suitable for quick detection of carriers in PXE families and sporadic patients. Another positive feature of this strategy is the value of the advanced DHPLC method in the molecular diagnosis of PXE. While DHPLC has been used in mutation screening for a number of other inherited diseases,[14,15,19,21,31] no data was yet available for ABCC6. In our study, apart from a set of known mutations, two new mutations and several new polymorphisms (data not shown) were detected by optimised DHPLC protocols.

In conclusion, our data suggest that DHPLC can efficiently identify known mutations in the *ABCC6* gene. The interpretation of the results of molecular analysis for genetic counselling remains challenging. One of obstacles is the fact that the inheritance mode of PXE is not yet completely understood. We discussed this issue in chapter 5.

Inheritance mode of PXE

How to interpret a molecular diagnosis is a task for the clinical molecular geneticists and genetic counsellors. Currently, it is uncertain if autosomal dominant (AD) inheritance exists in PXE. The key approach to this issue is to establish a relationship between clinical features in PXE, inheritance patterns and types of mutations. In chapter 5, we discussed this issue by reviewing the literature and our own familial segregation data. In our literature search we excluded reports on AD PXE when no detailed data were available on pedigrees or parental consanguinity. We found that three families in three different reports, that had PXE in two successive generations were proposed as AD inheritance.[1,8,25] However, in two of these pedigrees, partial phenotypic expression of PXE was described in only eye or skin tissue in one generation. This could result from mild expression in carriers of AR inheritance.[3] Pseudo-dominant inheritance could not be eliminated in these two families either. Segregation in another rare family (chapter5, family1) described by Pope could most likely be explained due to AD inheritance with reduced penetrance.[25]

In our own data (76 families), we focused on three putative AD families. In one family, AD segregation was plausible. In the other two families, AR inheritance with pseudo-dominance could not be excluded.

In conclusion, pseudo-dominance could be an explanation for most AD PXE reports in the literature and in our study. Indeed, the apparent high carrier frequency of the most common mutation R1141X (1 in 120) (Hu X. et al. unpublished data) in the Dutch population suggests that pseudo-dominance may exist as a common phenomenon in PXE. In addition, it could also be attributed to clinical manifestations present in carriers of PXE.[3, 27] We conclude that AD inheritance in PXE may exist, but is much rarer than previously thought and probably has a low penetrance.

R1141X is associated with coronary artery disease

We have demonstrated in chapter 3 that a founder mutation, R1141X, was the most common mutation in PXE patients in the Netherlands. We found a significant association between the R1141X mutation and premature coronary artery disease (CAD), which is described in chapter 5. We performed a case-control study with 441 patients with premature coronary artery disease (CAD) and 1057 age and sex matched controls free of CAD. The R1141X mutation was found in

3.2% of CAD patients. The prevalence of R1141X in the control group was 0.8%. Statistical analysis showed a significant difference between the patient and control groups (? < 0.001). This was a first report that the presence of the R1141X mutation in the *ABCC6* gene was associated with a sharply increased risk for premature CAD.

No obvious skin changes or angioid streaks were described in an AR PXE family with a 3775 del T, which results in a premature stop codon.[6] In this family, 40-50% of the heterozygous subjects only showed cardiovascular symptoms.[30] Together with the findings in our current study, a possible explanation of this phenomenon is that a heterozygote for *ABCC6* nonsense mutations may have a predisposition to cardiovascular disease.

Localization and distribution of the ABCC6 protein

Tissue expression of ABCC6 was first studied in humans by Kool et al with a RNase protection assay.[16] High *ABCC6* mRNA levels were found in liver and kidney and low levels in most other tissues. No *ABCC6* mRNA was detected in a few tissues like brain, testis, bladder, heart and spleen. A recent real time RT-PCR study largely confirmed this distribution pattern.[17] We did detect *ABCC6* mRNA in tissues frequently affected in PXE patients such as skin, retina, and blood vessel walls, but the levels were much lower than those in liver. Remarkably, in the tissue fraction of the RPE containing choroid and Bruch's membrane, no detectable ABCC6 mRNA was found.[6]

Recently, transport by ABCC6 of several molecules including glutathione conjugates, leukotrien-C 4 (LTC4) and N-ethylmaleimide S-glutathione (NEM- GS) in humans has been documented.[12] Alterations of the extracellular matrix in PXE were identified in several studies, such as abnormal amounts of proteoglycans accumulated around and within calcificated elastic fibers,[2,23,24] as well as abnormal amounts of glycosaminoglycans or their alternative products in urine.[20,21,29] However, it is not clear how this transport defect eventually results in such changes and calcification of elastic fibers in PXE affected tissues.

We generated polyclonal and monoclonal antibodies (Mabs) and used them successfully for the localization and distribution of the ABCC6 protein in chapters 3 and 7. A distribution study was carried out in frozen human tissue sections and appreciable staining with ABCC6 Mabs was shown in kidney and liver sections. No distinct and consistent ABCC6 staining was found in retinal sections. It is possible

that the expression of ABCC6 in the retina is so low that it can only be detected by RT-PCR analysis[6] but falls below the detection level of immnohistochemistry. In addition, no visible staining of ABCC6 in skin sections was seen either. In contrast, we did detect ABCC6 with the same Mabs in cultured fibroblasts (chapter 3) in which ABCC6 was located in the cytoplasma. This discrepancy may be attributed to technical aspects or to different epitope exposure between in vivo and in vitro models.

Further and conclusive localization studies indicated that ABCC6 was located at the basolateral membrane of the proximal tubules in the kidney. In the liver, the anti-ABCC6 Mabs strongly stained the basolateral membranes of hepatocytes. By double staining, it became clear that ABCC6 and MRP2 were, respectively, located at the basolateral and canalicular membranes. The localization of ABCC6 on the basolateral membrane suggests that ABCC6 may potentially pump substrate(s) from the liver back into the blood (Fig.2). A primary defect of ABCC6 in liver and kidney could result in abnormal levels of ABCC6 substrates in the blood, which could affect the elastic fiber assembly at specific sites in the body. On the other hand, lower expression of ABCC6 in affected tissues in PXE[6] could have an effect on PXE phenotype when there is a defect in the local ABCC6 expression. Consequently, PXE phenotype might be attributed to indirect calcification either or both systemic or local ABCC6 defects.

Clearly, the localization of ABCC6 in the retina has important consequences for the development of a functional hypothesis. Recently, Abcc6 mRNA expression in mouse retina was observed with in situ hybridisation and a RNase protein assay.[5] In situ hybridisation showed that Abcc6 localizes in the retinal ganglion cell layer, inner nuclear layer, outer nuclear layer, and the inner segments of the photoreceptors. The latter was further confirmed by immunohistochemical analysis with Abcc6 rabbit polyclonal antibodies. However, final proof of the exact distribution and localization of Abcc6 in the mouse retina can best be demonstrated in our ongoing studies of the Abcc6 knock-out model.

In summary, our clinical and experimental data as well as functional transport studies suggested that loss of transport function of ABCC6 protein is the primary cause of PXE (Fig 2).

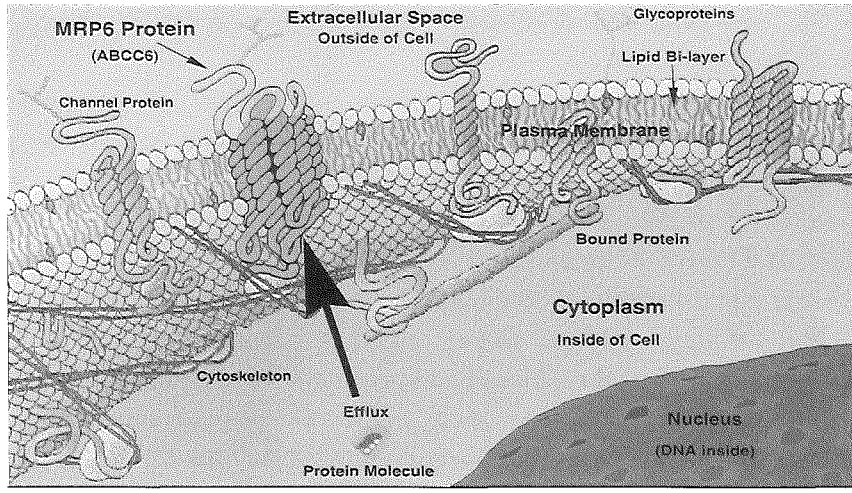


Figure 2: Functional model of ABCC6. High expression of ABCC6 is observed in the basolateral membrane in human liver and kidney. It suggests that ABCC6 functions as an efflux pump to transport substrate(s) from the liver back to the blood. A deficiency of specific ABCC6 substrate(s) transported to the blood may possibly affect a wide range of extracellular matrices in the entire body.

Further prospects

Molecular pathology of PXE in a knock-out mouse model

We constructed a complete knock-out PXE mouse model with a deficiency in the Abcc6 gene, which will be a useful tool to study the physiological and pathological functions of ABCC6 (unpublished results) . We generated this model by deletion of the first NBF and confirmed Abcc6 deficiency in an Abcc6 (-/-) mouse by genotyping and immunohistochemical staining liver sections with polyclonal antibodies (data not shown). The first point of interest will be to determine whether a phenotype similar to PXE in humans will develop in the model. Further investigation of the phenotype will include:

- Fundus examination by funduscopy and when indicated fluorescein angiography.
- Post mortem histological studies with von Kossa stain in skin, retina, blood vessels, lung, kidney and liver.
- Involvement of the cardiovascular system by ultrasonography.

- The expression and distribution patterns in the retina of Abcc6 by polyclonal Abcc6 antibodies.
- Abnormalities of metabolic pathways by analysis of components deposited in affected elastic fibers and the sulfated polyanions in urine by gas chromatograph–mass spectrometer (GS-MS) analysis.
- Influence of diet on the severity of disease by supplying calcium and magnesium, in different dosages.
- Gene expression patterns in the RPE by microarray analysis.

For long-term studies, factors including environment and genetic background that will affect the variation and penetrance on the phenotype will be evaluated in inbred mice. Together, these studies will generate important new insights into ABCC6 function and dysfunction and form the basis for future studies aimed at the development of a therapy for PXE.

Establishing the relationship of phenotype and genotype

While mutation data indicated that about 80% out of PXE patients carried at least one affected *ABCC6* allele, little is known about the potential correlations between genotype and phenotype. The phenotype of PXE shows considerable variation both within and between families. It is not clear, except for mutations in the *ABCC6* gene, whether other unknown gene(s) and/or environment factors, such as diet, play a role in the variable expression of PXE phenotype. A feasible strategy for future studies is to focus on one or more frequent mutations, such as R1141X, by combining clinical, family, and experimental data. Given the fact that most *ABCC6* mutations in PXE create premature termination of codons due to nonsense and frameshift mutations, information from R1141X may be applicable to a broader spectrum of other mutations.

Lack of informative familial data may be still a main obstacle for clarifying AD inheritance. The traditional mechanisms used to determine mode of inheritance (pedigree analysis combined with skin biopsy and eye examination) may be inconclusive since carriers, heterozygous for a single copy of an AR gene, may exhibit characteristic changes resulting in the appearance of two generations. Also the clinical classification system needs further improvement. Haplotype analysis with additional microsatellite markers and single-nucleotide polymorphisms within and flanking the *ABCC6* gene may be of help in clarifying segregation patterns in these families.

Characterizing functional consequences of mutations in ABCC6

To date, approximately 57 different *ABCC6* mutations in PXE patients have been reported (Chapter2, Fig1). Loss of transport function due to either a defect protein or no functional protein at all was suggested as disease mechanism as described in chapter 3 and by Ilias et al (Ilias, 2002). In further studies, implication of other DNA mutations should also be studied on transcript and/or on protein level. Studies on transcripts may increase our knowledge on gene mutations influencing splicing processes or even on a number of *ABCC6* gene changes within introns which have been missed in routine screening so far. Information from transcripts may also apply to those mutations occurring in the first nine exons where pseudogenes are located. Immunocytochemical staining with ABCC6 specific Mabs in cultured fibroblasts should be performed in selective families. It may produce data on functional deficits on the protein level. In addition, characterization of mutations from different regions of ABCC6 protein in fibroblasts or cell lines might offer the possibility to elucidate the correlation between affected elastic fibers and the *ABCC6* gene. Transport studies using specific mutation(s) identified in a family, such as R1459C with putative AD inheritance, may solve questions about molecular pathology and type of segregation. Finally, a mouse model lacking Abcc6, generated by our group, will be an essential tool for the full elucidation of the etiology of PXE.

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Summary/Samenvatting

Summary

This thesis describes research to elucidate the molecular pathology of pseudoxanthoma elasticum (PXE), from phenotype to genotype. PXE is an autosomally inherited disorder of connective tissue, affecting the skin, Bruch's membrane of the eye, and cardiovascular system. The most apparent clinical features of PXE is the skin manifestation. Ocular features include angioid streaks, peau d'orange, and comet-like streaks in the retina. Disability in PXE is usually the result of vascular complications, among which loss of visual acuity due to hemorrhage and scarring in the centre of the retina, the macula lutea.

PXE is either sporadic or usually segregates as an recessive disorder. Also dominant inheritance has been described. PXE is caused by mutations in the *ABCC6* (ATP-binding cassette, subfamily C, member 6) gene encoding a transmembrane protein.

The aim of this thesis was outlined in a brief introduction on PXE in chapter 1.1. The history of PXE was described in chapter 1.2 in which we summarized clinical, histopathological, and molecular aspects of PXE by reviewing the literature over the period from 1966 to 2002.

I started my PhD research by performing mutation analysis and setting up an *ABCC6* gene mutation database of Dutch PXE patients (chapters 2 and 4). Twenty mutations including 14 new ones were identified in 76 patients with PXE. The mutation detection rate in 152 *ABCC6* alleles was 58.6%. In 76 patients, we found 61 of these (80.3%) to carry at least one disease-causing allele. Two frequent mutations, R1141X and a deletion in exons 23-29 constituted respectively 39.3% and 19.1% of all mutant alleles. These *ABCC6* mutations tended to be located in the cytoplasmic part of the protein and to form three clusters in nucleotide binding fold 1 and 2 (respectively NBF1 and NBF2), and the eighth cytoplasmic loop. The unique distribution pattern of *ABCC6* mutations and their potential implication for function were discussed. Given that up to 80% of mutations were predicted to lead to a premature stop codon, we presume that a complete loss of functional ABCC6 protein is the mechanism underlying PXE.

In chapter 3, we characterized the most frequent mutation, R1141X, in 16 patients and families. A founder effect was proved by haplotype analysis with markers within and flanking the *ABCC6* gene. Next, we disclosed that the stability of the aberrant *ABCC6* mRNA was largely reduced. In patients homozygous for

R1141X very little or no *ABCC6* mRNA was detected. In subjects heterozygous for R1141X, *ABCC6* mRNA from the wild type allele was expressed much stronger compared to the mutant allele. Furthermore, immunocytochemical staining of cultured dermal fibroblasts with ABCC6-specific antibodies did not detect ABCC6 protein in patients homozygous for R1141X. These results indicate that the R1141X mutation is a null mutation, which causes premature chain termination and/or results in a non-functional protein. Loss of functional protein of ABCC6 may result from the unstable expression of mutant transcripts. The latter may be triggered by nonsense-mediated decay and may be involved in the pathogenesis of PXE.

In chapter 4, we aimed at setting up an efficient and rapid strategy for screening known *ABCC6* mutations in PXE patients. Using this strategy, 78.7% of mutant alleles can be identified for each case within a single week. It is useful for the detection both of new sporadic cases and members of known affected families. In addition, we applied the advanced denaturing high performance liquid chromatography (DHPLC) method for the first time in the detection of *ABCC6* mutations.

The association of the R1141X mutation and coronary artery disease was investigated in patients with premature coronary artery disease (441 cases) and a control group (1057 cases) as presented in chapter 5. The prevalence of R1141X in the patient group was 3.2%, which was four times higher than that in control subjects. The significant difference between patient and control groups ($p < 0.001$) suggested that the R1141X mutation plays a role in premature coronary artery disease as a risk factor. Whether the *ABCC6* gene is involved in cardiovascular disease through elastine degeneration or a different pathway remains to be further clarified.

The mode of inheritance in PXE was examined by reviewing the literature and our own data, as described in chapter 6. We found that the inheritance in most PXE families, reported in the literature to segregate in an autosomal dominant (AD) fashion, actually could be better explained by pseudodominance than by AD segregation. In only one of our own three putative AD PXE families, the inheritance pattern could reasonably be explained by AD inheritance. Therefore, AD inheritance in PXE may exist, but if so, it is rare.

To increase the knowledge of the function of the *ABCC6* gene, we studied its cellular localization and distribution (chapter 7). First, we made specific monoclonal

antibodies against ABCC6. We localized ABCC6 in the basolateral membranes of hepatocytes and proximal tubules of the kidney of normal humans by using immunohistochemical staining. While high expression of ABCC6 was found in normal kidney and liver, low, or no expression was found in multiple other tissues, including those affected by PXE (skin; retina). The latter finding suggested that PXE is in fact a systemic disease and that is not caused by local defect.

Together, the results presented in this thesis indicate that the PXE phenotype is mainly caused by loss of ABCC6 transport function. ABCC6 may function as an efflux pump to transport specific substance(s) necessary for extracellular matrix assembly. Defective transport activities of ABCC6 may be responsible for the elastic fiber accumulation at specific sites in the body, such as Bruch's membrane. Further study on a knockout mouse model generated in our group may contribute to elucidate ABCC6 function.

Samenvatting

Dit proefschrift beschrijft onderzoek naar de moleculaire pathologie van pseudoxanthoma elasticum (PXE). PXE is een autosomaal overervende bindweefselaandoening welke de huid, de membraan van Bruch in het netvlies evenals het hart-/vaatsysteem kan aantasten. Huidveranderingen zijn het meest duidelijke klinische verschijnsel van PXE. De meest voorkomende oogsymptomen zijn peau d'orange (sinaasappelschil-achtige, fijne hyper- en hypopigmentaties van het netvlies in de buurt van de gele vlek), angioide en komeet-achtige strepen in het netvlies. Functiebeperking door PXE is meestal het gevolg van vaatafwijkingen. In het oog leiden deze tot bloedingen en littekens in de gele vlek, resulterend in daling van de gezichtsscherpte.

PXE komt meestal sporadisch voor of erft in een autosomaal recessieve vorm over hoewel ook autosomaal dominante overerving beschreven is.
Het fundamentele kenmerk van PXE is de ophoping van gefragmenteerde elastine vezels in de aangedane weefsels met daarop afzetting van mineralen. PXE wordt veroorzaakt door mutaties in het *ABCC6* (ATP-bindend domein, subfamilie C, lid 6) gen dat codeert voor een eiwit dat tot nu toe onbekende stoffen door celmembranen heen transporteert.

Het doel van het proefschrift wordt in een korte inleiding over PXE in hoofdstuk 1.1 uitgelegd. De geschiedenis van het PXE-onderzoek wordt beschreven in hoofdstuk 1.2 waarbij de klinische, histopathologische en moleculaire aspecten van PXE, zoals beschreven in de literatuur tussen de periode 1966 tot 2002, werden samengevat.

Aan het begin van deze studie verrichtte ik mutatieanalyses en zette ik een *ABCC6* genmutatiedatabank op van PXE-patiënten uit Nederland (hoofdstukken 2 en 4). In 76 patiënten met PXE werden 20 mutaties, waaronder 14 nieuwe, gevonden. In 58,6% van de 152 *ABCC6* allelen konden mutaties opgespoord worden. Van de 76 patiënten hadden er 61 (80.3%) minstens één gemuteerd *ABCC6* allel. Twee frequent voorkomende *ABCC6* mutaties, R1141X en een deletie in exonen 23-29, vormden respectievelijk 39.3% en 19.1% van alle mutante allelen. Deze mutaties waren meestal gelokaliseerd in het cytoplasmatische deel van het eiwit en vormden drie groepen, gelocaliseerd in de nucleotide bindende plooien (folds) NFB1, NFB2 en in de achtste cytoplasmatische lus. Het unieke verdelings-

patroon van de *ABCC6* mutaties en hun potentiële betekenis voor de functie van ABCC6 werden besproken. Wij nemen aan dat totaal verlies van functionerend ABCC6 eiwit het belangrijkste mechanisme is dat aan PXE ten grondslag ligt omdat ongeveer 80% van de mutaties leken te leiden tot een vroegtijdig stopcodon.

In hoofdstuk 3 hebben wij de meest voorkomende mutatie, R1141X, in 16 patiënten en families nader gekarakteriseerd. Wij konden een vooroudereffect aantonen door haplotype analyse met intragene en *ABCC6* flankerende merkers. Ook vonden wij dat de stabiliteit van het abnormale *ABCC6* R1141X mRNA sterk verminderd was. In patiënten die homozygoot waren voor R1141X werd heel weinig of geen *ABCC6* mRNA aangetoond. In personen heterozygoot voor R1141X, werd *ABCC6* mRNA van het wild type allel veel sterker tot expressie gebracht dan mRNA van het mutante allel. Ook toonde immunocytochemische kleuring van gekweekte huidfibroblasten met specifieke monoclonale antilichamen tegen ABCC6, geen tekenen van aanwezigheid van een (ingekort) eiwit in patiënten die homozygoot waren voor R1141X. Deze resultaten bevestigen dat de R1141X mutatie een nulmutatie is die leidt tot een prematuur eindigen van het afschrijfproces en een niet-functionerend eiwit. Verlies van functionerend ABCC6 eiwit kan het gevolg zijn van de onstabiele expressie van gemuteerde transcripten en deze instabiliteit, in gang gezet door nonsense geregelde mRNA afbraak, kan betrokken zijn bij de pathogenese van PXE.

In hoofdstuk 4 beschrijven wij een efficiënt en snelle moleculair diagnostische strategie om *ABCC6* mutaties in PXE-patiënten te screenen. Door middel van deze strategie konden 80.9% van de mutante allelen voor iedere patiënt in een enkele week geïdentificeerd worden. Deze strategieontwikkeling is belangrijk voor het opsporen van zowel nieuwe als sporadische personen met PXE en voor familieleden van bekende families. Daarbij pasten wij voor het eerst de geavanceerde DHPLC methode toe voor het opsporen van *ABCC6* mutaties.

De associatie tussen R1141X mutatie en ziekte van de coronaire slagaders werd onderzocht in 441 patiënten die vroegtijdig een aandoening van de coronaire slagaders kregen en een controlegroep van 1057 personen, zoals weergegeven in hoofdstuk 6. De prevalentie van de R1141X mutatie in de patiëntengroep bedroeg 3.2%, viermaal hoger dan in de controlegroep. Het significante verschil tussen de patiënten- en controlegroepen ($p < 0.001$) suggereert dat de R1141X mutatie een risicofactor is voor vroegtijdig coronair slagaderlijden. Of de *ABCC6* mutatie

betrokken is bij cardiovasculaire ziekten door elastine fragmentatie, of via een ander mechanisme, moet nog opgehelderd worden.

De overervingswijze in PXE werd onderzocht via literatuurstudie en via ons eigen databestand, zoals vermeld in hoofdstuk 6. Voorheen werd aangenomen dat in 10 % - 15 % van de PXE families dominante overerving plaats vond. Wij vonden echter, dat de overerving in de meeste PXE-families, beschreven als autosomaal dominant, eerder verklaard kon worden door pseudo-dominantie bij hoge allel frekwentie in de bevolking dan door autosomaal dominante overerving. In onze drie families met mogelijke autosomaal dominante overerving, kon bij nadere analyse maar in één redelijkerwijze worden verklaard dat de overerving autosomaal dominant had plaatsgevonden. Om die reden denken wij nu dat autosomaal dominante overerving in PXE wel kan bestaan, maar dat dit zeer zeldzaam is.

We bestudeerden de cellulaire lokalisatie en verdeling van het ABCC6 eiwit om onze kennis van het werkingsmechanisme te vergroten (hoofdstuk 7). Eerst maakten we specifieke monoklonale en polyklonale antilichamen tegen ABCC6. Met behulp van immunohistochemische kleuringen werd ABCC6 gelokaliseerd in de basolaterale membranen van levercellen en proximale niertubuli in gezonde mensen. De weefsels die het vaakst in PXE zijn aangedaan, zoals huid en het netvlies, kleurden niet in de vriescoupes. Dit zou er op kunnen duiden dat PXE in feite een systemische ziekte is, en niet zozeer door een lokale weefselafwijking ontstaat.

Concluderend kan men zeggen dat de resultaten van dit proefschrift er op wijzen dat het PXE fenotype altijd optreedt bij volledig verlies van de ABCC6 transportfunctie. Indien er een partieel verlies is van ABCC6 transportfunctie, zoals bij heterozygote *ABCC6* mutatie dragers, dan kan het PXE phenotype wel of niet ontstaan, afhankelijk van tot nu nog onbekende omgevingfactoren en/of andere genen. ABCC6 werkt waarschijnlijk als een moleculaire pomp die specifieke stoffen vanuit lever en nier terugpompt in het bloed. Die stoffen zijn mogelijk betrokken bij de aanmaak en afbraak van extracellulaire matrix, en/of de ophoping van abnormale elastinevezels op diverse speciale plaatsen elders in het lichaam, zoals de huid en de membraan van Bruch. Wij verwachten dat nieuwe studies aan de hand van het door ons gemaakte PXE muizen knock-out model zullen bijdragen aan de verdere ontrafeling van de functie van ABCC6.

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