New insights into the pathogenesis of pseudoxanthoma elasticum and related soft tissue calcification disorders by identifying genetic interactions and modifiers

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INTRODUCTION

Mutations in ABCG6, a gene encoding for the ABC transporter protein 6 of subfamily C, formerly known as multidrug resistance-associated protein 6 (MRP6), are the cause of pseudoxanthoma elasticum (PXE; Kool et al., 1999; Bergen et al., 2000; Le Saux et al., 2000; Ringpfeil et al., 2000; Miksch et al., 2005; Schulz et al., 2006). PXE is an autosomal recessive disorder characterized by soft tissue calcification primarily in the skin, Bruch’s membrane in the retina and the vessel walls (Neldner and Struk, 2002). To date, more than 300 – mostly unique – PXE-associated ABCG6 mutations have been identified (http://www.ncbi.nlm.nih.gov/lovdb/home.php?select_db=ABCG6). Despite large epidemiological studies including more than 500 well-characterized PXE patients, no genotype-phenotype correlations have been discovered so far. Moreover, there is a great clinical variability between PXE patients, even within families with more than one person affected. This heterogeneity between patients raises the question of whether factors, e.g., environmental or genetic background, contribute to PXE manifestation, disease progression, and severity. The assumption is supported by the description of PXE-related diseases (Vanakker et al., 2007) as well as clinical overlaps with other rare monogenetic syndromes and common cardiovascular disorders (Trip et al., 2002). A recent publication in this special issue on soft tissue mineralization focused on the clinical phenotype of PXE and its parallels to other cardiovascular diseases (Lefthériotis et al., 2013).

Here, we summarize current knowledge of the genetics underlying PXE and PXE-related disorders characterized by soft tissue calcification.

Keywords: PXE, rare disease, mineralization, calcification, genetic interaction, genetic modifier

SCREENING OF THE ABCG6 GENOME

Screening of the adenosine triphosphate binding cassette transporter protein subfamily C member 6 gene (ABCG6) in pseudoxanthoma elasticum (PXE) revealed a mutation detection rate of approximately 87%. Although 25% of the unidentified disease alleles underlie deletions/insertions, there remain several PXE patients with no clear genotype. The recent identification of PXE-related diseases and the high intra-familiar and inter-individual clinical variability of PXE led to the assumption that secondary genetic co-factors exist. Here, we summarize current knowledge of the genetics underlying PXE and PXE-related disorders based on human and animal studies. Furthermore, we discuss the role of genetic interactions and modifier genes in PXE and PXE-related diseases characterized by soft tissue calcification.

MUTATIONAL ANALYSIS OF THE ABCG6 GENE

Recently, the PXE candidate gene, ABCG6 (MIM #606324), was identified and mutations in this gene were found to cause PXE (exemplarily: Le Saux et al., 2000). The ABCG6 gene contains 31 exons and encodes a 165 kDa transmembrane transporter of 1509 amino acids. The physiological function of ABCG6 is still unknown. To date, more than 300 causative ABCG6 mutations have been identified. These include missense, nonsense, and splice site mutations, as well as deletions and insertions. According to current studies, PXE seems to be inherited in an exclusively autosomal recessive mode (Plump et al., 2004; Miksch et al., 2005; Pfendner et al., 2007). Hence, PXE patients are homozygous or compound heterozygous carriers of ABCG6 mutations. Most mutations observed in ABCG6 are unique and the majority of them are located within cytoplasmic domains of ABCG6. The most frequent mutation found in PXE patients is a nonsense mutation in exon 24, p.R1141X (c.3421C>T, rs72653706), which is found in approximately 25% of the European patient population. A larger deletion of exons 23–29 (c.2996_4208del) represents the most common mutation (25%) in American PXE patients. The mutation detection rate lies between 80 and 90%. Consequently, in up to 20% of patients clinically diagnosed with PXE, only one or no ABCG6 mutations were detected. As a consequence of methodological limitations, small ABCG6 deletions/insertions in homozygous state could be missed by direct sequencing approaches. Costrop et al. (2010) uncovered about 25% of the missing alleles as deletions of various sizes by performing multiplex ligation-dependent probe amplification (MLPA). These findings emphasize the importance of screening...
for deletion/insertions in the molecular diagnostics of PXE. Nevertheless, there still remain PXE patients with an incomplete ABCC6 genotype. Consequently, other genetic causes or environmental factors may be involved in PXE manifestation.

**GENETIC INTERACTIONS**

The recent identification of inherited disorders related to PXE and characterized by soft tissue calcification suggests multiple genetic factors. Vanakker et al. (2007) described a novel PXE-like syndrome (MIM #560842), which is inherited in an autosomal recessive mode, but caused by mutations in the GGCX gene, encoding γ-glutamyl carboxylase. Patients present with generalized redundant skin folds, a mild retinopathy and a coagulation defect of vitamin K-dependent clotting factors (Vanakker et al., 2007). Similar cases had already been reported in the early 1990s (Le Courvaizer-Pieto et al., 1996). Histological examination of a skin biopsy reveals fragmentation and calcification of mid-dermal elastic fibers identical to PXE. However, ultrastructural analysis by electron microscopy showed differences in elastic fiber mineralization: in PXE-like syndrome, the calcification occurred in the periphery of the elastic fiber rather than in the fiber core. Li et al. (2009a, b) described two different families with combined features of PXE and a vitamin K-dependent coagulation deficiency. In both studies mutational screening of ABCC6, GGCX, and VKORC1 revealed two putative pathogenic mutations in GGCX in patients presenting with distinct skin lesions and a coagulation factor deficiency. Interestingly, the authors observed similar skin lesions in the mother and her twin sister with no evidence of a coagulation disorder (Li et al., 2009a). Mother and paternal aunt were identified as compound heterozygous carriers of the recurrent GGCX mutation p.R1141X and a missense mutation in GGCX (p.S300F). The authors suggested that the skin phenotype is because of digenic inheritance.

Generalized arterial calcification of infancy (GACI, MIM #208000) is another rare autosomal recessive trait characterized by soft tissue calcification (Rutsch et al., 2003). Mutations in ENPP1, encoding for ectonucleotide pyrophosphatase 1, are the primary cause of GACI. Patients present with extensive hydroxyapatite deposition in large and medium-sized arteries, leading to arterial stenosis and visceral ischemia. A recent study reported a family with two brothers (Le Boulanger et al., 2010). One was suffering from PXE while the other died at 15 months of age from a condition similar to GACI. Molecular analysis of ABCC6 and ENPP1 in this family revealed two pathogenic ABCC6 mutations in heterozygous state as the primary cause of disease. Pathogenic ENPP1 mutations were not identified except for two common ENPP1 sequence variations. It remains speculative whether these lead to a more severe disease progression. Following on from these results, Nitschke et al. (2012) screened ABCC6 and reanalyzed ENPP1 for pathogenic mutations in a larger cohort of patients with clinically proven GACI (n = 92). The authors found 30 patients who did not carry any ENPP1 mutations. Pathogenic ABCC6 mutations in homo- or compound heterozygosity were found as the primary cause for GACI in 15 patients (Nitschke et al., 2012). The fact that mutations in ENPP1 and ABCC6 manifest in overlapping disease phenotypes of GACI and PXE suggests similar metabolic pathways are involved in the pathogenesis.

The significance of genetic interactions is further supported by experimental studies with Abcc6−/−;Ggcx+/− mice in which the onset of ectopic calcification was delayed, whereas Abcc6−/−;Ggcx−/− mice presented with accelerated mineralization (Li and Uitto, 2010).

**DEFINITION OF MODIFIER GENES**

The "genetic co-factor" concept is not new, and was introduced by Haldane as early as 1941 (Haldane, 1941). A primary reason for searching for genetic factors modifying disease is to provide information on the disease course and to improve therapies (if available). Several definitions of so-called "modifier genes" exist in the literature. We define a modifier gene as a gene that when "mutated," is insufficient on its own to cause disease, but when coupled with another genetic mutation, produces or enhances its pathogenesis. "Modifier genes" have been detected for cystic fibrosis, another monogenic disorder caused by an ABC transporter defect (ABCL7 gene) with variable disease onset and progression (Curtis, 2005; Drummond et al., 2005).

**SELECTION OF CANDIDATE GENES**

In PXE several metabolic pathways seem to be affected, e.g., the regulation of biological calcification, extracellular matrix (ECM) remodeling and lipid transport and biosynthesis. Sequence variations in genes regulating these pathways may be involved in the development and clinical course of PXE.

Regulation of biological calcification:

Ectopic calcification is the result of a complex interplay between stimulating and inhibitory proteins and metabolites. One candidate gene for PXE susceptibility is secreted phosphoprotein 1 (SPP1, formerly known as osteopontin). SPP1 fulfills important functions in the regulation of biological calcification and was also found to be a constitutive component of elastic fibers in order to prevent them from calcification (Baccarani-Contrri et al., 1994). A study by Aherrahrou et al. described a dramatic up-regulation of SPP1 expression in mice suffering from dystrophic cardiac calcification (DCC, Aherrahrou et al., 2004). The Abcc6 gene was identified as the potential candidate gene for DCC in mice (Korff et al., 2006). SPP1 is a predominantly transcrip- tional regulated gene and the SPP1 promoter is highly conserved among the human, murine, and porcine genes (Hijjawi et al., 1994). Several polymorphisms in SPP1 were shown to affect SPP1 expression (Giacopelli et al., 2004; D’Alfonso et al., 2005; Hummelshoj et al., 2006). We found that the c.−1748G, the c.153_156insG and the c.244,245insTG alleles appear to be significantly more common in PXE patients (Hendig et al., 2007). The polymorphism
c.155_156insG generates a RUNX2 (run-related transcription factor 2) binding site closed to a second RUNX2 binding site in the SPP1 promoter. RUNX2 is an essential transcriptional regulator of osteoblast differentiation and bone formation. Conclusively, we interpreted three SPP1 promoter polymorphisms and the haplotype combining these disease-associated alleles as a putative genetic risk pattern for PXE.

Fetuin-A, was found to be a major systemic inhibitor of calcification (Jahnen-Dechent et al., 2001). Carriers of fetuin-A genotype 2 have been shown to have the lowest serum fetuin-A concentration (Stenvinkel et al., 2008). Even though we did not observe an association between PXE manifestation and fetuin-A genotype, it could be speculated that fetuin-A genotype 2 is an additional disease-promoting risk factor (Hendig et al., 2006).

Matrix gla protein (MGP) is a calcification inhibitor acting locally. MGP was also found in the circulatory system, where it is part of the so-called calcification proteins, together with fetuin-A and hydroxypapitate. The importance of MGP in preventing pathological calcification is supported by Mgp-deficient mice, which develop severe arterial calcification (Minouree et al., 1999). Analysis of the MGP promoter polymorphism frequencies revealed one MGP haplotype to be a potential protective genetic co-factor in PXE (Hendig et al., 2008).

**ECM-re remodeling and oxidative stress**

Several studies reported an increased ECM remodeling in skin of PXE patients. In this context increased expression of proteases, a mild oxidative stress, and an altered proteoglycan metabolism was detected. The latter resulted in increased accumulation of proteoglycans in the ECM, and in changes in the structure and composition of urinary glycosaminoglycans (Longas et al., 1986; Maccari et al., 2003; Korrest et al., 2004; Volpi and Maccari, 2005).

Many of the alterations observed in PXE could be explained by oxidative stress, for instance ECM remodeling. We found a correlation between genotype and age of disease onset for polymorphisms in the genes catalase (CAT, c.262C>T), superoxide dismutase 2 (SOD2, c.47C>T), and glutathione peroxidase 1 (GPX1, c.593C>T), encoding essential antioxidant enzymes (Zarbock et al., 2007). Furthermore, the age of disease onset was inversely correlated with the number of mutated alleles, indicating a cumulative effect on the time of PXE onset.

Elevated production of MMP2 (matrix metallopeptidase 2) in PXE fibroblasts and increased levels of MMP2 and MMP9 in serum from PXE patients were shown (Quaglino et al., 2005; Dickmann et al., 2009). Increased MMP expression may at least partly result from genetic variation. We have previously shown that variations in MMP2 are a genetic co-factor for PXE (Zarbock et al., 2010).

We propose that xylanase/transfase I (XYLT, XT-I), as the initial and most important enzyme in proteoglycan biosynthesis, and XT-II, as a highly homologous protein, might contribute to the increased ECM synthesis rate in PXE. As most XT-I is secreted into the ECM, XT activity was proposed as a diagnostic marker for the determination of enhanced proteoglycan biosynthesis and tissue destruction (Götting et al., 2007). Moreover, elevated XT activity was found in sera from PXE patients, the higher the proteoglycan biosynthesis rate (Götting et al., 2005). We further showed that three sequence variants in the XT/L2 gene result in a severe disease course of PXE (Schön et al., 2006).
been identified as heterozygous carriers of only one ABCC6 mutation. Taken together, these results underline the assumption that ABCC6 mutations on a single allele might determine a mild PXE phenotype. This hypothesis might have great impact considering ABCC6 as genetic modifier of other rare monogenic disorders (GACI), or common disease (stroke, myocardial infarction, and CAD). Wang et al. (2012) and EVI1 expression of the frequent ABCC6 p.R1268Q variant (rs150468; c.3736-334A>G) and the p.R1141X variant (c.3803G>A) point toward new disease entities which seem to be phenotypically similar to PXE or GACI at first sight but differ in yet unknown features. Genetic modifiers are discussed as concomitant factors contributing to the course of the disease. The search for modifier genes is difficult but worthwhile being performed in view of better knowledge of biological pathways affected by the disease. Moreover, numerous studies have identified such genes in mice. The availability of various mouse strains carrying the same ABCC6 sequence variation associated with more or less severe soft tissue calcification provides an excellent starting point for functional studies and the investigation of interactions between ABCC6 and specific modifier genes (Berndt et al., 2012).

The recent identification of modifier genes for PXE underscores the importance of the analysis of gene-gene environment interactions in understanding the development of complex phenotypes such as PXE. As genetic modifiers are known to alter the course and expression of disease, their gene products become interesting targets for therapeutic intervention. Discovery of genetic modifiers for example affecting the success of anti-neovascular therapy could be, in the future, a key issue to obtain a personalization of therapy and to avoid unnecessary costs in PXE. Nevertheless, replication studies analyzing the new association of modifier genes in other PXE patient cohorts are essential to determine whether these modifier genes are indeed a genetic risk factor for PXE and related disorders. Moreover, the importance of consistent phenotype measures and complementary study designs cannot be overemphasized. Here, family studies and sibling analysis may help to estimate the contribution of genetic and non-genetic factors.

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REFERENCES
Abrahamsen, Z., Attems, S. B., Kaczmarek, P. M., Israt, A., Koff, S., Doehring, L. C., et al. (2004). A locus on chromosome 7 determines dramatic up-regulation of osteopontin in dystrophic cardiac calcification in mice. Am. J. Pathol. 164, 1379–1387. doi: 10.1016/S0002-9440(04)65224-9
Amourouz, L., Vogl, S., Ghadri, D., Paquale-Rossetti, L., Zanone, C., Cetti, G., et al. (2004). High levels of desmosines in urine and plasma of patients with pseudoxanthoma elasticum. Eur. J. Clin. Invest. 34, 136–146. doi: 10.1111/j.1365-2265.2004.01306.x
Amoros, S., E., and Beckmann, J. J. S. (2006). Mendelian disorders deserve more attention. Nat. Rev. Genet. 7, 277–282. doi: 10.1038/nrg1826
Bacarani-Cortesi, M., Vincenzi, D., Cicchetti, F., Mori, G., and Paquale-Rossetti, I. (1994). Immunohistochemical identification of abnormal constituents in the dermis of pseudoxanthoma elasticum patients. Eur. J. Histol. 58, 111–123.
Bergen, A. A., Plump, A. S., Schraermann, E. I., Terry, S., Brenning, M., Danovaro, H., et al. (2000). Mutations in ABCC6 cause pseudoxanthoma elasticum. Nat. Genet. 25, 226–231. doi: 10.1038/76109
Bertoli, A., Li, Q., Potter, C. S., Liang, Y., Silva, K. A., Kennedy, Y., et al. (2012). A single nucleotide polymorphism in the ABCC6 gene associates with connective tissue mineralization in mice similar to targeted models for pseudoxanthoma elasticum. J. Clin. Invest. 122, 155–166. doi: 10.1172/JCI54120
Costrop, L. M., Vanacker, O. O., Van Laer, L., Le Sauv, O., Martin, L., Chassang, N., et al. (2010). Novel deletions causing pseudoxanthoma elasticum instability of the ABCC6 region. J. Hum. Genet. 55, 112–117. doi: 10.1523/JNEUROSCI.2630-09.2009
Cuttin, G. R. (2005). Modifier genetics: cystic fibrosis. Annu. Rev. Genomics Hum. Genet. 6, 237–269. doi: 10.1146/annurev.genomics.6.080604.162254
D’Alfonso, S., Barizzone, N., Giorgetti, P., Mancino, S., Greco, D., Cicchetti, F., et al. (2004). Polymorphisms in the ABCC6 region are associated with plasma lipoprotein profile. Hum. Genet. 115, 329–334. doi: 10.10039/art.20809
Diestelmann, U., Zurbock, B., Herzig, D., Nolka, C., Kleiss, K., and Götting, C. (2009). Elevated levels of matrix metalloproteinases MMP-2 and MMP-9 in pseudoxanthoma elasticum patients. J. Med. Genet. 46, 1065–1070. doi: 10.1136/jmg.2009.069475
Dreum, M. L., Konstan, M. D., Schlachter, A., Hamid, P., Rast, P., Zori, F., et al. (2000). Genetic modifiers of lung disease in cystic fibrosis. N. Engl. J. Med. 342, 355–363.
Giacomelli, F., Marconato, K., Potoccro, A., Catani, P., Canini, S., Kowetry, G., et al. (2004). Polymorphisms in the osteopontin promoter affect its transcriptional activity. Physiol. Genomics 19, 87–96. doi: 10.1152/physiogenomics.00138.2004
Glim, M., Zayetz, J. D., Fenger, R. P., Hida, R. G., Lamy, B. P., and Elinson, P. (2013). An update on the ocular...
Hornstrup, L. S., Tybjærg-Hansen, A., Hijiya, N., Setoguchi, M., Mattiazzi, P., Zarbock, R., Szliska, C., Hendig, D., Arndt, M., Szliska, C., Haldane, J. B. (1941). The relative... of stimulated proteoglycan biosynthesis in pseudoxanthoma elasticum. J. Lipid Res. 25, 387–392. doi: 10.1194/jlr.P008268

Peluso, G. M., Demisse, S., Collins, D., Miel, D. B., Gabrielli, S. B., Cupples, L. A., et al. (2010). Common genetic variation in multiple metabolic pathways influences susceptibility to low HDL-cholesterol and coronary heart disease. J. Lipid Res. 51, 3524–3532. doi: 10.1194/jlr.P008268

Fusini, E., Van Lieshout, A. M., Terry, S. R., Voulaire, M. D., Voulaire, M. P., Esler, M. K., et al. (2007). Mutation detection in the ABCG5 and ABCG8 genes in a family with pseudoxanthoma elasticum: evidence for drusenoid 6-sulfate aberrations. Clin. Genet. 71, 227–232. doi: 10.1111/j.1399-0004.2006.00921.x

Bellocchio, A., Fresa, R., Guerra, M., et al. (2004). A novel Lys479Asp mutation in the human matrix Gla protein causes Keutel syndrome. J. Mol. Med. 82(3):175–182.

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Hendig et al. (2005). Dermal fibroblasts from pseudoxanthoma elasticum patients have raised MMP-2 degradation potential. Biochem. Biophys. Acta 1741, 42–47. doi: 10.1016/j.bbmbi.2004.09.012

Ringpfeil, F., Lebwohl, M. G., and Garbisa, S. (2005). Pseudoxanthoma elasticum mutations in the MRFP gene encoding a transmembrane ATP-binding cassette (ABC) transporter. Proc. Natl. Acad. Sci. U.S.A. 97, 6001–6006. doi: 10.1073/pnas.100214997

Bunck, C., Rad, N., Vaingankar, S., Telkst, M. K., Suk, A., Hohne, W., et al. (2003). Mutations in ENPP1 are associated with ‘idiopathic’ infantile arterial calcification. Nat. Genet. 34, 379–381. doi: 10.1038/ng1001

J. Invest. Dermatol. 127, 581–587. doi: 10.1038/sj.jid.5500310

Wongman, J. J., Ha, X., Tan, H., Berger, A. A., Trip, M. D., Smulders, Y. M., Wegman, J. J., Hu, X., Boot, J. M., Tan Brink, J. B., et al. (2002). Frequent mutation of the ABCC6 gene (R1141X) is associated with a strong increase in the prevalence of coronary artery disease. Circulation 106, 773–775. doi: 10.1161/01.CIR.0000042021.27913.L0

Vasikirat, O. M., Martin, L., Gheduzzi, D., Levy, B. F., Levy, B. L., Guerci, J. I., et al. (2007). Pseudoxanthoma elasticum-like phenotype with cutis laxa and multiple coagulation factor deficiency represents a separate genetic entity. J. Invest. Dermatol. 127, 581–587. doi: 10.1097/jid.0b013e3180368e0b

Volp, N., and Maccari, F. (2005). Composition of urinary glycosaminoglycans in a patient with pseudoxanthoma elasticum and familial Mediterranean fever. Clin. Chim. Acta 359, 207–209. doi: 10.1016/j.cca.2005.04.008

Wongman, J. J., Ha, X., Tan, H., Berger, A. A., Trip, M. D., Kastelein, J. J., et al. (2005). Patients with premature coronary artery disease who carry the ABCC6 R1141X mutation have no pseudoxanthoma elasticum phenotype. J. Clin. Cardiol. 100, 399–395. doi: 10.1016/j.jcc.2004.07.012

Zarbock, R., Hendig, D., Sizaka, C., Klouek, K., and Götting, C. (2007). Pseudoxanthoma elasticum: genetic variations in antioxidant genes are risk factors for early disease onset. Clin. Chim. Acta 374, 154–170

Zarbock, R., Hendig, D., Sizaka, C., Klouek, K., and Götting, C. (2005). Analysis of MMP2 promoter polymorphisms in patients with pseudoxanthoma elasticum. Clin. Chim. Acta 359, 207–209. doi: 10.1016/j.cca.2005.04.008

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