GENETICS OF STRUCTURAL SKIN DISORDERS

Heritable Ectopic Mineralization Disorders: The Paradigm of Pseudoxanthoma Elasticum

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ECTOPIC MINERALIZATION IN SKIN DISEASES

Skin diseases characterized by aberrant accumulation of mineral deposits comprise a heterogeneous group of disorders, often associated with inflammation (Sprecher, 2010). Examples of such conditions are systemic autoimmune disorders, for example, dermatomyositis, lupus erythematosus, and systemic sclerosis. Localized inflammatory lesions, such as those in acne and skin cancers, can also develop localized foci of mineralization. The mechanisms of ectopic mineralization in these relatively common conditions are largely unknown. However, clues to the mineralization processes affecting the skin have emerged from studies on rare heritable single gene disorders with profound mineralization of the skin. The paradigm of such mineralization disorders is pseudoxanthoma elasticum (PXE), a multi-system disease with protean manifestations in the skin, the eyes, and the cardiovascular system (Neldner, 1988). In fact, the significant milestones of progress made in understanding this disorder serve as examples of the power of molecular genetics towards identification of the candidate genes and pathogenic mutations, with translational implications (Uitto *et al.*, 2010, 2011).

PXE—THE PARADIGM OF ECTOPIC MINERALIZATION DISORDERS

PXE was initially described as a clinical syndrome with characteristic skin findings, ocular involvement, and cardiovascular manifestations (Neldner, 1988). The cutaneous lesions, i.e., small yellowish papules, which tend to coalesce into leathery and inelastic skin, demonstrate accumulation of pleiomorphic elastic structures in mid-dermis, which by diagnostic histopathologic examination reveals progressive mineralization. Although the cutaneous findings primarily present a cosmetic problem and do not interfere with the normal life activities, they signify the risk for development of ocular and vascular complications that can be quite debilitating, with considerable morbidity and occasional premature mortality. The skin findings are associated with ocular involvement, manifesting with angioid streaks and progressive loss of visual acuity, occasionally resulting in blindness. The cardiovascular manifestations include hypertension, intermittent claudication, occasional bleeding from the gastrointestinal vessels, and early myocardial infarcts, reflecting calcification of the arterial blood vessels.

CANDIDATE GENES AND GENE DISCOVERY

As the histopathology of skin in PXE reveals abnormal elastic structures, the genes that participate in the synthesis and assembly of the elastic fibers were initially considered as a candidate gene/protein system for mutations in this disorder. This included the elastin gene on chromosome 7q, as well as a number of elastin-associated microfibrillar proteins, such as fibrillins 1 and 2, fibulins 2, 3, and 4, and lysyl oxidases. However, with cloning of the corresponding genes, linkage analyses systematically excluded these chromosomal regions as the sites of ''the PXE gene'' (Christiano *et al.*, 1992; Raybould *et al.*, 1994). The first milestone of molecular genetics on PXE consisted of a collaborative teamwork, organized primarily by PXE International, the premiere patient advocacy organization, which established linkage of the PXE gene to the short-arm of chromosome 16 (Le Saux *et al.*, 1999; Cai *et al.*, 2000). The critical interval initially consisted of ~500 kb of human genome that contained four annotated genes, and examination of the human genome database revealed that none of these genes had an obvious connection to extracellular matrix of connective tissue in general or the elastic fiber network in particular. Another milestone consisted of systematic sequencing of these candidate genes, which resulted in identification of the *ABCC6* gene as the one harboring mutations in PXE (Bergen *et al.*, 2000; Le Saux *et al.*, 2000; Ringpfeil *et al.*, 2000; Struk *et al.*, 2000). This gene encodes a transmembrane efflux transporter protein, ABCC6, a member of the family of multi-drug resistance proteins (MRP6). The gene consists of 31 exons spanning ~75 kb of the human genome on chromosomal region 16p13.1.

MUTATION SPECTRUM AND GENETIC HETEROGENEITY

Following the initial identification of *ABCC6* as the PXE gene, more than 300 distinct mutations representing over 1,000 mutant alleles have been encountered in patients with PXE from varied ancestral and ethnic backgrounds (Chassaing *et al.*, 2005;

Miksch *et al.*, 2005; Pfendner *et al.*, 2007; Li *et al.*, 2009a). The types of mutations include missense and nonsense mutations, intronic mutations causing missplicing, small deletions or insertions within exons resulting in frameshift of translation, as well as large deletions spanning part or the entire coding region of *ABCC6*, and sometimes even including flanking genes. Two recurrent mutations of high frequency have been identified, one of them being p.R1141X in exon 24, which accounts for ~30% of all pathogenic PXE mutations. In addition, a recurrent deletion of exons 23–29 (del23–29; pA999-S1403del) has been found in at least one *ABCC6* allele in 20% of US and 12% of European patients. With the expansion of the *ABCC6* mutation database, these genetic lesions can be used for confirmation of the clinical diagnosis, carrier detection, and presymptomatic identification of affected individuals. Furthermore, early identification of the disease has aided in increased surveillance of the clinical complications, allowing prevention, and undoubtedly improving the quality of life of the affected individuals.

Although it is clear that mutations in the *ABCC6* gene underlie most cases with classic forms of PXE, defects in additional genes have recently been discovered to result in PXE-like cutaneous findings. A particularly intriguing observation, with potential pathomechanistic implications for PXE, was demonstration that patients with vitamin K-dependent coagulation factor deficiency due to mutations in the *GGCX* gene, can also have PXE-like cutaneous findings (Vanakker *et al.*, 2007; Li *et al.*, 2009b). In addition to characteristic cutaneous lesions similar to those seen in PXE, i.e., small yellowish papules, these patients demonstrate excessive folding and sagging of the skin with loss of recoil. These patients were initially described as having combined clinical features of both PXE and cutis laxa. However, the cutaneous lesions in these patients depict characteristic mineralization of dermal elastic structures similar to PXE. More recently, another gene, *ENPP1*, which underlies generalized arterial calcification of infancy, a severe mineralization disease affecting the cardiovascular system that often leads to early demise of the affected individuals within the first few years of life, has also shown to be associated with PXE-like cutaneous findings (Li *et al.*, 2012; Nitschke *et al.*, 2012).

The *GGCX* gene encodes an enzyme responsible for γ-glutamyl carboxylation of Gla-proteins, such as vitamin K-dependent coagulation factors and matrix Gla-protein, the latter being a powerful anti-mineralization factor expressed in peripheral connective tissues. Most of these patients show inactivating missense mutations in both alleles of *GGCX*, clinically manifesting with both PXE-like cutaneous findings and bleeding tendency. In some cases, the *GGCX* mutations were shown to be heterozygous in combination with a heterozygous *ABCC6* mutation, manifesting with cutaneous findings consistent with PXE but without coagulation disorder, suggesting digenic inheritance of PXE in this family (Li *et al.*, 2009c). These milestones attest to the presence of an intricate mineralization/anti-mineralization network in the skin and indicate that mutations in different genes involved in ectopic tissue mineralization can result in PXE-like phenotypes.

MODEL SYSTEMS AND PATHOMECHANISMS

An early, somewhat puzzling observation on PXE was that the *ABCC6* gene is expressed primarily in the liver and to a lesser extent in the proximal tubules of kidneys, and only at very low level, if at all, in tissues demonstrating ectopic mineralization (Belinsky and Kruh, 1999; Scheffer *et al.*, 2002). The pathomechanistic details of PXE remain unclear, and in particular, the identity of the molecules transported from liver to the circulation by ABCC6 remains to be disclosed. However, significant progress has been made in understanding the molecular nature of PXE. The critical milestone towards understanding this disease was development of transgenic animal models with features of PXE (Gorgels *et al.*, 2005; Klement *et al.*, 2005). Specifically, targeted ablation of the mouse *Abcc6* gene results in a phenotype that recapitulates features of human PXE, including late-onset (5–6 weeks after birth), progressive mineralization of connective tissues. The mineral deposits accumulating in peripheral connective tissues of these mice have been shown to consist of calcium and phosphate forming hydroxyapatite crystals, similar to that in patients with PXE (Walker *et al.*, 1989; Kavukcuoglu *et al.*, 2012). This mouse model has served as a platform to study the consequences of *Abcc6* mutations utilizing skin grafting and parabiotic pairing model systems (Jiang *et al.*, 2009, 2010a). These experiments have provided evidence that PXE is a metabolic disorder in which absence of ABCC6 transporter activity in the liver results in deficiency of circulating anti-mineralization factors, which in wild-type mice prevent precipitation of calcium/phosphate complexes under normal homeostatic conditions (Figure 1). One such circulating factor has been suggested to be vitamin K or its derivatives, such as reduced vitamin K–glutathione conjugate, which is required for activation of the matrix Gla-protein by γ-glutamyl carboxylase, a vitamin K–dependent enzyme (Borst *et al.*, 2008). This hypothesis has been tested in *Abcc6*$^{-/-}$ mice by feeding them with excessive amounts of vitamin K (Brampton *et al.*, 2011; Gorgels *et al.*, 2011; Jiang *et al.*, 2011). The results indicated that vitamin K supplementation did not prevent ectopic mineralization in the *Abcc6*$^{-/-}$ mouse model, suggesting that peripheral tissue mineralization in PXE is not a result of deficiency in vitamin K concentration in tissues.

The nature of the substrate transported by ABCC6 in the liver has also been examined by development of an *in vitro* cell-based transporter system (Ilias *et al.*, 2002). In this system, insect Sf9 cells are transfected with human ABCC6 expression vector, and the cells are then used to make inside-out vesicles that can be used in transport assays. This assay system has revealed that ABCC6 can transport anionic small molecular weight compounds, but specifically, ABCC6 does not transport vitamin K–glutathione conjugate (Fülöp et al., 2011).

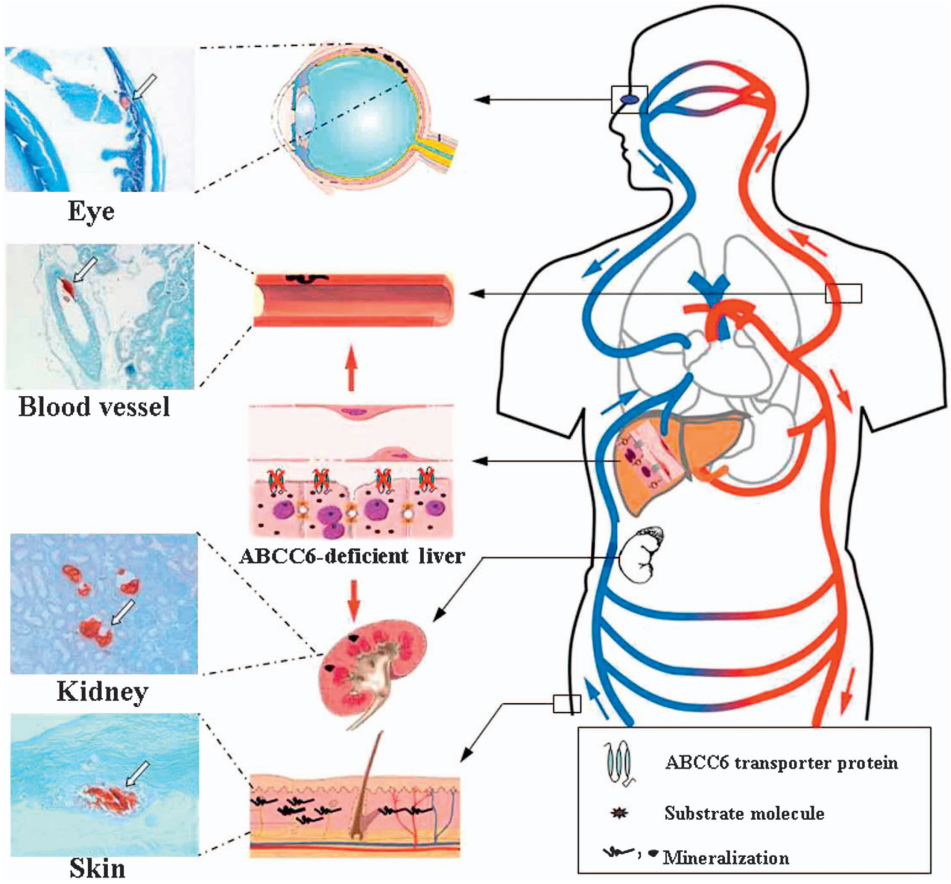

Figure 1. Conceptual illustration of the proposed metabolic hypothesis of PXE. Under physiologic conditions, the ABCC6 protein is expressed in high levels in the liver, presumably transporting critical metabolites to the circulation (right panel). In the absence of ABCC6 transporter activity in the liver, changes in the concentration of such substrate molecules in the circulation can take place, and the changes result in mineralization of a number of tissues, such as the eye, the arterial blood vessels, the kidney, and the skin (middle panel). The presence of mineralization is detected in transgenic *Abcc6*$^{-/-}$ mice that recapitulate features of human PXE, by Alizarin red stain (left panel).

TREATMENT PROSPECTS FOR PXE

Two lines of milestone studies have suggested potential treatment modalities for PXE, currently an intractable disorder. One of those studies is predicated on prevention of mineralization that could be helpful in lessening the clinical manifestation of PXE irrespective of the pathomechanisms. Specifically, a set of experiments has suggested that supplementation of diet with magnesium will prevent the deposition of mineral in connective tissue (LaRusso *et al.*, 2009; Gorgels *et al.*, 2010; Li and Uitto, 2010). Specifically, experiments using the *Abcc6*$^{-/-}$ animal model have shown that supplementation of the diet with magnesium up to 5-fold completely prevents the development of ectopic mineralization up to 6 months of age. Conversely, diet that is low in magnesium and high in phosphate has been shown to accelerate the mineralization process in PXE mice (LaRusso *et al.*, 2008; Li and Uitto, 2010; Jiang and Uitto, 2012). Thus, these studies examining the precise role of different mineral components in the diet, using the preclinical animal models, have led to development of clinical trials to test the feasibility of this approach to treat patients with PXE.

Another potential way of preventing ectopic mineralization revolves around the supplementation of anti-mineralization factors to the circulation. One of such molecules is fetuin-A, a powerful anti-mineralization factor that is able to prevent aberrant mineralization under normal physiologic calcium and phosphate homeostatic conditions (Jahnen-Dechent *et al.*, 1997). The possibility of using fetuin-A is strengthened by demonstration that concentrations of fetuin-A in serum of patients with PXE as well as in *Abcc6*$^{-/-}$ mice are reduced (Hendig *et al.*, 2006; Jiang *et al.*, 2007), and in fact, overexpression of fetuin-A in the liver of *Abcc6*$^{-/-}$ mice has been shown to result in lessening of the mineralization (Jiang *et al.*, 2010b). Additional strategies aim at regeneration of liver by introduction of allogeneic stem cells with capability to differentiate into hepatocytes, which could result in restoration of functional ABCC6 transporter activity. An extension of this strategy would be liver transplantation or a partial lobe replacement as a means to restore the ABCC6 activity.

An intriguing possibility is that distinct mutations in the *ABCC6* gene have different pathomechanistic

consequences with respect to ABCC6 activity, which manifest as ectopic mineralization. Although most of the pathogenic lesions are nonsense mutations resulting in reduced or absent synthesis of the ABCC6 protein or missense mutations that result in inactivation of the transporter function, certain mutations have been shown to impair the intracellular trafficking of transporter-competent ABCC6 proteins (Ilias *et al.*, 2002). Specifically, two missense mutations, p.R1138Q and p.R1314W, have been shown in *in vitro* assays to retain significant transporter activity, yet both mutant proteins display endoplasmic reticulum localization *in vivo*. The cellular localization of p.R1314W mutant was improved by treatment with 4-phenylbutyrate, a chemical chaperone that facilitates trafficking of misfolded proteins, thus potentially rescuing its physiological function (Le Saux *et al.*, 2011). This work demonstrates the feasibility of the *in vivo* rescue of cellular maturation of some ABCC6 mutants depending on the specific mutation. Collectively, development of molecular strategies can provide new avenues for treatment and eventual cure of PXE, a currently intractable disease.

CONFLICT OF INTEREST

The author states no conflict of interest.

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