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Variants in genes encoding pyrophosphate metabolizing enzymes are associated with Pseudoxanthoma elasticum

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ABSTRACT

Objectives: Pseudoxanthoma elasticum (PXE) is a rare hereditary disorder characterized by progressive calcification and fragmentation of elastic fibers. Because of the great clinical variability between PXE patients the involvement of modifier genes was recently suggested. Therefore, we investigated the association of single nucleotide variants (SNVs) in selected candidate genes known to regulate cellular pyrophosphate metabolism.

Design and methods: We used RLFP analyses to evaluate the distribution of SNVs in alkaline phosphatase (ALP), ectonucleotide pyrophosphatase 1 (ENPP1) and ankylosis (ANKH) in DNA samples from 190 German PXE patients and 190 age- and sex-matched healthy controls. Statistical analyses were performed using Fisher exact test and Bonferroni correction.

Results: The screening revealed three different SNVs in three genes, which were associated with PXE. The SNV c.1190-65C > A (rs1780329, minor allele frequency (MAF) patients: 0.17; controls: 0.11; $P = 0.04$) in the ALP gene was significantly more frequent in PXE patients. Furthermore, PXE was highly associated with ANKH p.A98A genotype TT ($P = 0.0012$), although the MAF was not different between patients and controls. After correction for multiple testing according to the Bonferroni method, one SNV in the ENPP1 gene (c.313 + 9G > T, rs7773477) remained significantly associated with PXE with significantly higher MAF values in the patient cohort (MAF: 0.04 vs. 0.00; $P = 0.0024$) and a high association with PXE susceptibility (OR 27.96).

Conclusion: Polymorphisms in ALP, ENPP1 and ANKH are important genetic risk factors contributing to PXE.

Introduction

Progressive calcification and fragmentation of elastic fibers, which affect skin, eyes and the cardiovascular system, are major characteristics of Pseudoxanthoma elasticum (PXE, OMIM 264800). PXE, a rare hereditary disorder, is caused by mutations in ABCC6 (ATP-binding cassette subfamily C, member 6), a gene encoding for an ABC-transporter protein also known as multidrug resistance-associated protein 6 (MRP6) [1]. The clinical course of PXE is highly variable, with age at disease onset and the number and magnitude of its symptoms differing considerably among patients [2]. It has been speculated that other genes, so-called modifier genes, as well as environmental factors might contribute to the expression and severity of PXE. In the last years the relevance of this hypothesis could be confirmed by identification of modifier genes for PXE [3–7]. The encoded proteins are involved in the biosynthesis of glycosaminoglycans, response to oxidative stress and regulation of biological calcification, which were discussed as playing key roles in the pathomechanisms underlying PXE. Biological calcification was previously reported to be a passive process, but recent evidence suggests a complex regulation involving multiple steps and mediators. Due to progressive calcification of elastic fibers as a hallmark of PXE and the fact that the calcification process is actively regulated, we suggest further modifier genes for PXE. Candidate genes for PXE susceptibility are tissue-nonspecific alkaline phosphatase (ALP), inorganic pyrophosphate (PP_i) channel progressive ankylosis protein homolog (ANKH) and transmembrane ectonucleotide pyrophosphatase 1 (ENPP1). We have just recently shown the essential role of PP_i as the central regulatory metabolites preventing matrix calcification in PXE [8]. PP_i acts as an inhibitor of basic calcium phosphate crystal growth in the extracellular matrix [9]. The concentration of PP_i is controlled by ENPP1, which generates AMP and PP_i from ATP, by the PP_i

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channel ANKH and ALP [9–11]. ALP cleaves PP_i and phosphorylated osteopontin (OPN), whereby these two calcification inhibitors become inactive and P_i is released. P_i is a component of hydroxyapatite crystal deposition and supports extracellular matrix mineralization.

Investigations into ENPP1 metabolism led to a correlation between PXE and another rare disorder known as generalized arterial calcification of infancy (GACI, OMIM 208000). GACI is characterized by a progressive calcification of the internal elastic lamina, fibrotic myointimal proliferation of muscular arteries, and resultant arterial stenosis in the neonatal period [12]. It was observed that PXE and GACI can be caused by mutations in either ABCC6 or ENPP1 [13]. The two other candidate genes, ALP and ANKH, also induce calcification associated diseases, but none of them were already linked to PXE. Mutations in the ALP gene cause ankylosing spondylitis (AS), a form of chronic arthritis characterized by inflammatory response and pathological mineralization that primarily affects young adults. Hypophosphatasia, a second disease distinguished by defective bone mineralization and deficiency of serum ALP activity, is a rare autosomal recessive inborn error of metabolism. ANKH mutations are linked to autosomal-dominant chondrocalcinosis (CC), which manifests in joint pain and arthritis caused by the deposition of calcium-containing crystals within articular cartilage [14]. These candidate genes play important roles in the regulation of matrix calcification. The aim of our current study was to investigate selected single nucleotide variants (SNVs) in ALP, ANKH and ENPP1 as modifiers for PXE manifestation and disease severity, because PP_i seems to be a central regulatory metabolite preventing matrix calcification in PXE.

Materials and methods

Patients and controls

The study was approved by the ethics commission of the Ruhr University of Bochum Faculty of Medicine, located in Bad Oeynhausen. All patients provided their written informed consent to participate in the study. EDTA-anticoagulated whole blood samples were obtained from 190 German PXE patients and from 190 age- and sex-matched healthy controls. The control cohort consists of Westphalian blood donors, who were healthy and had no symptoms suggestive for PXE. Patients were recruited between 2001 and 2008 from the multidisciplinary referral center for patients with PXE, Bethesda Hospital, Freudenberg, Germany. Clinical data were collected retrospectively by reviewing patient charts from the Departments of Dermatology, Ophthalmology and Medicine. The diagnosis of PXE in all patients was consistent with the reported consensus criteria [15]. We analyzed 56 male and 134 female patients with a mean age of 46.9 years (±15.1 years) and an age of diagnosis of 31.8 years (±16.4 years). Clinical characteristics were as follows: 169 (89%) patients with skin involvement,157 (83%) patients with involvement of the eyes, 79 (42%) patients with involvement of the vascular system, 58 (31%) patients with hypertension, and 29 (15%) patients with heart involvement (e.g. myocardial infarction, cardiomyopathy). Of note, these numbers do not exclude subclinical or faint disease manifestations. For instance, ocular alterations sometimes may be detected best using advanced imaging technologies [16–18] which, however, have not been used for phenotyping the cohort described herein.

DNA preparation

Genomic DNA was extracted from 200 μl EDTA blood using the QIAamp blood kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA was stored at −20 °C.

ABCC6 genotyping

Mutational analysis of the ABCC6 gene and the common c.3421C > T mutation were performed as described previously [19,20]. A multiplex PCR was used to detect the frequent 16-kb deletion c.EX23_EX29del as reported by Hu et al. [21]. To detect ABCC6 deletions/insertions, which can be missed by direct sequencing, multiplex ligation-dependent probe amplification (MLPA) was applied in PXE patients with an incomplete genotype [22].

Polymerase chain reaction

PCR primers were designed using the published sequence (GenBank accession no. ALP NG_008940, ANKH NG_008273, ENPP1 NG_008206). PCR was performed in 25 μl reaction volume, containing 25 pmol of each primer (Biomers, Ulm, Germany), 1 U HotStar Taq DNA polymerase (Qiagen, Hilden, Germany) in 1 × reaction buffer including 2.5 mM $MgCl_2$ (Qiagen, Hilden, Germany), 0.25 mM of each dNTP (Solis Biodyne, Tartu, Estonia) and 5 μl DNA template. The PCR conditions were as follows: initial denaturation at 95 °C for 2 min, 35 cycles of denaturation at 95 °C for 1 min, annealing for 1 min, extension at 72 °C for 1 min and final extension at 72 °C for 10 min. The primer sequences, annealing temperatures and sizes of the PCR products are summarized in Supplement 1.

Allele-specific PCR

The polymorphism c.294C > T (rs17251667) was analyzed using allele-specific PCR. PCR was performed twice as described above using primers with the specific sequence for each allele. Agarose gel electrophoresis was applied to detect the amplification products.

Restriction fragment length polymorphism analysis

The obtained DNA fragments were digested overnight with 1 U of either restriction endonuclease (Supplement 1) at the temperature recommended by the supplier and subsequently separated on a 1.5% agarose gel. All restriction endonucleases were purchased from New England Biolabs (Frankfurt, Germany).

Statistical analysis

Allele and haplotype frequencies were compared between cases and controls using Fisher's exact test. Correction for multiple testing was performed using the Bonferroni method. The chi-square test was used to examine whether the genotype distributions were within the Hardy–Weinberg equilibrium by comparing observed and expected genotypes. *P*-values of less than 0.05 were considered significant after Bonferroni correction. The association of each SNV with PXE was measured by the odds ratio (OR) and 95% CI. All tests were performed using GraphPad Prism 5.

Determination of linkage disequilibrium (LD) and haplotype blocks and frequencies were performed by Haploview 3.2 [23]. Haplotype blocks were defined according to the "spine of LD" setting in Haploview software, on the basis of each end marker of a block having a D′ value of more than 0.8.

Multifactor dimensionality reduction (MDR) software 3.0.2 was used for detecting gene–gene and gene–environment interactions [24]. Power calculations were performed using the program developed by Faul et al. [25].

Results

Association of single markers with PXE

Twelve single nucleotide variants (SNV) in the genes ALP, ANKH and ENPP1 were genotyped in DNA samples from 190 German PXE patients and 190 age- and sex-matched healthy controls. The SNVs were selected according to the following criteria: 1) functional relevance, 2) involvement in pathological calcification, and 3) at least 5% reported frequency.