SPPI Promoter Polymorphisms: Identification of the First Modifier Gene for Pseudoxanthoma Elasticum

Doris Hendig,1 Marius Arndt,1 Christiane Szliska,2 Knut Kleesiek,1 and Christian Göttig1*

Background: Progressive calcification and fragmentation of elastic fibers are characteristic hallmarks of pseudoxanthoma elasticum (PXE), which is caused by mutations in ABCC6 encoding multidrug resistance–associated protein 6 (MRP6). Because of the great clinical variability of PXE, secondary genetic risk factors are suspected to exist. We investigated whether SPPI (secreted phosphoprotein 1; previously OPN, osteopontin) promoter polymorphisms are associated with PXE.

Methods: We screened an ~2-kb region spanning the theoretical promoter of the SPPI gene for sequence variations by denaturing HPLC and direct sequencing in 93 PXE patients. Sequence variations with a prevalence >5% were genotyped in 93 age- and sex-matched healthy controls. Statistical and haplotype association analyses were performed using Fisher exact test, PHASE v2.1.1, and Haploview 3.2.

Results: Mutational screening revealed 9 different sequence variations. Three SPPI promoter polymorphisms (c.−1748A>G, c.−155_156insG, and c.244_245insTG) were significantly more frequent in PXE patients than in 93 age- and sex-matched healthy controls (P_corrected < 0.05 each). The odds ratios (95% CI) for PXE among carriers of the 3 alleles were, respectively, 2.16 (1.34–3.48), 2.41 (1.51–3.82), and 1.97 (1.23–3.15). Haplotype analysis of 6 SPPI promoter polymorphisms revealed 1 haplotype to be significantly reduced among PXE patients (P_corrected = 0.035, odds ratio 1.80, 95% CI 1.19–2.71).

Conclusions: Polymorphisms in the SPPI promoter are secondary genetic risk factors contributing to PXE susceptibility.

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Pseudoxanthoma elasticum (PXE)3 is an autosomal recessive disorder characterized by connective tissue alterations primarily in the skin, Bruch membrane in the retina, and the vessel walls (1). The disorder is histologically characterized by a progressive calcification and fragmentation of elastic fibers. PXE is caused by mutations in ABCC64 (ATP-binding cassette subfamily C, member 6), a gene encoding for the multidrug resistance–associated protein 6 (MRP6), an ATP-binding cassette (ABC) transporter protein with an as-yet-unnamed function (2–7). MRP6 is mainly found in the liver and kidneys, whereas little or no protein is observed in organs affected by PXE (2). The localization of MRP6 to the basolateral side in hepatocytes and kidney proximal tubules gives rise to the hypothesis that MRP6 is an export pump extruding substrates into the bloodstream (8, 9). Recent studies suggest that there might be a lack or an accumulation of factors, e.g., proteoglycans, reactive oxygen species, or systemic mineralization inhibitors such as fetuin-A, in the bloodstream of PXE patients owing to the absence of functional MRP6 (10–13). Such factors could also be involved in the expression of other components.

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finally resulting in the extracellular matrix alterations typical of PXE. Dermal fibroblasts deposit abnormal elastic fiber aggregates, interestingly not accompanied by mineralization, when cultured in the presence of PXE sera (14). Together, these data support the hypothesis that PXE is primarily a metabolic disorder.

The clinical course of PXE is highly variable. Although environmental influences may modify disease outcome, there might also exist secondary genetic variations (15). "Modifier genes" have been detected for cystic fibrosis, another heritable disorder with variable disease onset and progression (16). Cystic fibrosis is caused by mutations in CFTR (cystic fibrosis transmembrane conductance regulator), member 7 of the same ABC transporter subfamily as ABCC6. A candidate gene for PXE susceptibility is SPP1 (secreted phosphoprotein 1; previously OPN, osteopontin). SPP1 is a secreted, highly acidic phosphoprotein that is involved in immune cell activation, wound healing, and bone morphogenesis (17) and plays a major role in regulating mineralization processes in various tissues.

Increased SPP1 expression is often associated with pathological calcification. Furthermore, SPP1 is a constitutive component of human skin and aorta, where it is localized to the elastic fiber and hypothesized to prevent calcification in the fibers (18). Skin biopsies from PXE patients show higher expression of SPP1 than do samples from unaffected regions or from healthy individuals (19). SPP1 expression is increased in mice suffering from dystrophic cardiac calcification (20), leading to the suggestion that high SPP1 expression is influenced by a trans-activator gene, the Dyscalc1 locus on chromosome 7 (20). The mouse abcc6 gene was identified as a potential candidate gene in this region (21).

SPP1 is a predominantly transcriptional regulated gene, and the SPP1 promoter is highly conserved among different species (22). Several polymorphisms in the SPP1 gene affect SPP1 expression and have been associated with various disorders, e.g., systemic lupus erythematosus and arteriosclerosis (23–26).

We put forward the hypothesis that sequence variations in the SPP1 promoter region might account for the higher SPP1 expression observed in PXE patients and therefore promote disease outcome. We present data from a case-control association study on German PXE patients and an age- and sex-matched normal population with 6 sequence variations spanning the whole SPP1 promoter region.

Materials and Methods

Patients and Controls

EDTA-anticoagulated whole blood samples were obtained from 93 German PXE patients, ages 16 to 78 years, and from 93 age- and sex-matched healthy controls. The study cohort comprised 82 unrelated families with an apparently autosomal recessive or sporadic mode of inheritance of the PXE phenotype (Table 1). The diagnosis of PXE in all patients was consistent with the reported consensus criteria (27). The status of the PXE patients was determined by the presence of dermal lesions and ocular findings. The dermal lesions were histologically confirmed by the observation of mineralized elastic fibers in biopsy samples after von Kossa staining. To minimize interobserver variability, one medical specialist thoroughly questioned all study participants about personal diseases, organ involvement, and family history. The study was approved by the institutional review board, and the PXE patients provided informed consent.

DNA Extraction and Mutational Analysis in the Promoter Region of the SPP1 Gene

Genomic DNA was extracted from 200 μL EDTA-anticoagulated blood using the QIAamp blood reagent set (Qiagen) according to the manufacturer’s instructions. The entire regulatory region from −1864 to +317 of the SPP1 gene was initially amplified by PCR and then analyzed for sequence variations by partially denaturing HPLC (dHPLC). Nucleotide numbering refers to the SPP1 genomic DNA sequence (GenBank accession no. NT_016354), with the first nucleotide of exon 1 as the transcription initiation start site, referred to as nucleotide +1.

PCR was performed in a 50-μL reaction volume, containing ~65 ng genomic DNA, 25 pmol of each primer (Biomers), 1.5 units HotStar Taq DNA polymerase (Qiagen) in 1 × PCR buffer supplied with the enzyme, and 0.25 mmol/L of each dNTP (Promega). The primer sequences, annealing temperatures, and sizes of the PCR products are summarized in Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol53/issue5. The PCR conditions were as follows: initial denaturation at 95 °C for 15 min, 35 cycles of denaturation at 94 °C for 1 min, annealing for 1 min, and extension at 72 °C for 1 min, and a final extension at 72 °C for 15 min. dHPLC analysis was carried out on an automated HPLC device equipped

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Female</th>
<th>Male</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>65</td>
<td>28</td>
</tr>
<tr>
<td>Age, years &lt;sup&gt;a&lt;/sup&gt;</td>
<td>47.8 (14.5)</td>
<td>53.1 (12.8)</td>
</tr>
<tr>
<td>Age at disease onset &lt;sup&gt;a&lt;/sup&gt;</td>
<td>28.9 (16.1)</td>
<td>38.4 (15.9)</td>
</tr>
<tr>
<td>Current smoking &lt;sup&gt;b&lt;/sup&gt;</td>
<td>13 (20.0)</td>
<td>5 (17.9)</td>
</tr>
<tr>
<td>Organ involvement Number of organs &lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.1 (1.5)</td>
<td>3.5 (1.5)</td>
</tr>
<tr>
<td>Skin &lt;sup&gt;b&lt;/sup&gt;</td>
<td>61 (93.8)</td>
<td>23 (82.1)</td>
</tr>
<tr>
<td>Eyes &lt;sup&gt;b&lt;/sup&gt;</td>
<td>57 (87.7)</td>
<td>26 (92.9)</td>
</tr>
<tr>
<td>Cardiovascular tissue &lt;sup&gt;b&lt;/sup&gt;</td>
<td>20 (30.8)</td>
<td>14 (50.0)</td>
</tr>
<tr>
<td>Hypertension &lt;sup&gt;b&lt;/sup&gt;</td>
<td>20 (30.8)</td>
<td>8 (28.6)</td>
</tr>
<tr>
<td>Heart &lt;sup&gt;b&lt;/sup&gt;</td>
<td>8 (12.3)</td>
<td>9 (32.1)</td>
</tr>
<tr>
<td>Gastrointestinal &lt;sup&gt;b&lt;/sup&gt;</td>
<td>9 (13.8)</td>
<td>4 (14.3)</td>
</tr>
<tr>
<td>Kidney/urinary tract &lt;sup&gt;b&lt;/sup&gt;</td>
<td>6 (9.2)</td>
<td>7 (25.0)</td>
</tr>
<tr>
<td>Other &lt;sup&gt;b&lt;/sup&gt;</td>
<td>19 (29.2)</td>
<td>6 (21.4)</td>
</tr>
</tbody>
</table>

Data are <sup>a</sup> mean (SD) or <sup>b</sup> n (%).
with a DNA separation column (Wave System, Transgenic) as described (28). Oven temperatures and the initial and final concentrations of buffer B for dHPLC analysis are given in Table I of the online Data Supplement. PCR products of samples showing aberrant peaks were purified via exonuclease I (1 unit) and shrimp alkaline phosphatase (1 unit) treatment per 5 μL amplicon at 37 °C for 30 min. Inactivation of the enzymes was carried out at 80 °C for 15 min. The purified PCR product served as a template for mutation identification by direct sequencing on both strands. DNA sequencing of the PCR products was performed on an ABI Prism 310 capillary sequencer using the Big Dye Terminator v1.1 cycle sequencing reagent set (Perkin-Elmer Applied Biosystems), sequencer using the Big Dye Terminator v1.1 cycle sequencing reagent set (Perkin-Elmer Applied Biosystems), 3 μL purified PCR product, and 2.5 pmol of the same PCR primers used for amplification, in a total reaction volume of 20 μL. To exclude the possibility of amplification errors resulting from HotStar Taq DNA polymerase, PCR and inactivation of the enzymes was carried out at 80 °C for 15 min. The purified PCR product was used for the detection of the polymorphisms c.155_156insG, c.66T>G, and c.244_245 insTG. Samples initially genotyped as heterozygous were reanalyzed without mixing the sample with a known homozygous control. This approach allows for determination of whether the sample was heterozygous or homozygous for the variant. Genotyping for the polymorphisms c.1776T>C, c.1748A>G, and c.443C>T was performed by using mismatch PCR for construction of restriction sites, followed by digestion with an appropriate restriction enzyme. For the genotyping of c.616G>T, allele-specific PCRs were established. Primer sequences for mismatch and allele-specific PCR, annealing temperatures, sizes of the PCR products, and restriction enzymes are listed in Table 2 of the online Data Supplement. The restriction enzymes were obtained from New England Biolabs, and digestions were performed according to the manufacturer’s instructions.

**Genotyping of common SPP1 promoter polymorphism**

Sequence variations with a prevalence of >5% were genotyped in cases and controls by dHPLC or restriction fragment length polymorphism analysis. dHPLC analysis was used for the detection of the polymorphisms c.155_156insG, c.66T>G, and c.244_245 insTG. Samples initially genotyped as heterozygous were reanalyzed without mixing the sample with a known homozygous control. This approach allows for determination of whether the sample was heterozygous or homozygous for the variant. Genotyping for the polymorphisms c.1776T>C, c.1748A>G, and c.443C>T was performed by using mismatch PCR for construction of restriction sites, followed by digestion with an appropriate restriction enzyme. For the genotyping of c.616G>T, allele-specific PCRs were established. Primer sequences for mismatch and allele-specific PCR, annealing temperatures, sizes of the PCR products, and restriction enzymes are listed in Table 2 of the online Data Supplement. The restriction enzymes were obtained from New England Biolabs, and digestions were performed according to the manufacturer’s instructions.

**Statistical analysis and power calculations**

All polymorphisms were tested for confirmation with Hardy-Weinberg expectations in both cohorts analyzed. Allele and haplotype frequencies were compared between cases and controls using the Fisher exact test. The association of each polymorphism with PXE was measured by the odds ratio (OR) and 95% CI. Multiple testing correction was performed using the Bonferroni method. P <0.05 was considered significant after Bonferroni correction. All tests were executed with GraphPad Prism 4.0 (GraphPad Software). Power calculations were performed using the program developed by Skol et al. (29).

**Linkage disequilibrium structure and identification of haplotype blocks**

Determination of linkage disequilibrium (LD) and haplotype blocks and frequencies were performed by using 2 different validated programs, Haplovie v3.2 and PHASE v2.1.1 (30, 31), and comparing them. Haplotype blocks were defined according to the “spine of LD” setting in Haplovie software, which is on the basis of each end marker of a block having a D’ value of >0.8 (30).

**Results**

**Mutational analysis of the SPP1 promoter region**

We detected 9 sequence variations by performing dHPLC analysis of the SPP1 promoter sequence (Table 2). One of these variants, c.1625A>G, was newly identified, whereas the remaining polymorphisms were recently reported in the National Center for Biotechnology Information SNP (single nucleotide polymorphism) database or in the literature (23, 24). In our study, the major allele of polymorphism rs2853744 was c.616T in both cohorts analyzed; the major allele described in the National Center for Biotechnology Information SNP database is c.616G. The distribution of allele frequencies for all identified polymorphisms did not differ significantly from Hardy-Weinberg equilibrium. Three polymorphisms with a minor allele frequency of ≤5% (c.1776T>C, c.1625A>G, and c.1282A>G) were not analyzed further. The remaining 6 SPP1 promoter polymorphisms were genotyped in an age- and sex-matched control cohort.

**Association analysis of individual sequence variations**

Comparison of the allelic frequencies of the detected SPP1 promoter polymorphisms between PXE patients and healthy controls revealed the 4 variants c.1748A>G, c.155_156insG, c.66T>G, and c.244_245 insTG to be significantly more frequent in the PXE group (P <0.05 each) (Table 2). The association with PXE was significant for allele c.1748G (frequency in PXE patients vs controls: 0.333 vs 0.188, P = 0.0020), allele c.155_156GG (0.382 vs 0.204, P = 0.0002), allele c.66G (0.296 vs 0.177, P = 0.0102), and allele c.244_245TGTG (0.328 vs 0.199, P = 0.0066). After correction for multiple testing according to the Bonferroni method, only the 3 SPP1 promoter polymorphisms c.1748A>G, c.155_156insG, and c.244_245insTG remained significantly associated with PXE (Pcorrected <0.05) (Table 2). The presence of the disease-associated allele conferred an OR of 2.16 (95% CI 1.34–3.48) for allele c.1748G, 2.41 (1.51–3.82) for allele c.155_156GG, and 1.97 (1.23–3.15) for allele c.244_245TGTG.

To calculate the power of our sample size and genetic models, we used the algorithm of Skol et al. (29). The results indicate that our sample has a sufficient size to detect associations with a power of 79%–90% for a relative risk of ≥2.0 for the most frequent polymorphisms (minor
Table 2. Allele frequencies of SPP1 promoter polymorphisms detected in PXE patients and controls.

<table>
<thead>
<tr>
<th>Sequence variant*</th>
<th>Reference SNP*</th>
<th>Allele</th>
<th>Frequency in PXE patients*</th>
<th>Frequency in controls*</th>
<th>OR (95% CI)</th>
<th>P*</th>
<th>P_corrected*</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.-1776T&gt;C</td>
<td>rs29001511</td>
<td>T</td>
<td>180 (0.968)</td>
<td>181 (0.973)</td>
<td>1.21 (0.36–4.03)</td>
<td>1.0</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>6 (0.032)</td>
<td>5 (0.027)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.-1748A&gt;G</td>
<td>rs2728127</td>
<td>A</td>
<td>124 (0.667)</td>
<td>151 (0.812)</td>
<td>2.16 (1.34–3.48)</td>
<td>0.0020</td>
<td>0.012</td>
</tr>
<tr>
<td></td>
<td></td>
<td>G</td>
<td>62 (0.333)</td>
<td>35 (0.188)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.-1625A&gt;G</td>
<td>this study</td>
<td>A</td>
<td>185 (0.995)</td>
<td>ND</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>G</td>
<td>1 (0.005)</td>
<td>ND</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.-1282A&gt;G</td>
<td>ref. 32</td>
<td>A</td>
<td>185 (0.995)</td>
<td>ND</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>G</td>
<td>1 (0.005)</td>
<td>ND</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.-616G&gt;T</td>
<td>rs2853744</td>
<td>G</td>
<td>14 (0.075)</td>
<td>5 (0.027)</td>
<td>0.34 (0.12–0.96)</td>
<td>0.0569</td>
<td>0.3414</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T</td>
<td>172 (0.925)</td>
<td>181 (0.973)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.-443C&gt;T</td>
<td>ref. 31</td>
<td>C</td>
<td>93 (0.50)</td>
<td>110 (0.591)</td>
<td>1.45 (0.96–2.19)</td>
<td>0.0956</td>
<td>0.5736</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T</td>
<td>93 (0.50)</td>
<td>76 (0.409)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.-155_156insG</td>
<td>rs17524488</td>
<td>-</td>
<td>115 (0.618)</td>
<td>148 (0.796)</td>
<td>2.41 (1.51–3.82)</td>
<td>0.0002</td>
<td>0.0012</td>
</tr>
<tr>
<td>c.-66T&gt;G</td>
<td>rs28357094</td>
<td>T</td>
<td>131 (0.704)</td>
<td>153 (0.823)</td>
<td>1.95 (1.19–3.18)</td>
<td>0.0102</td>
<td>0.0612</td>
</tr>
<tr>
<td>c.-244_245insTG</td>
<td>rs5860110</td>
<td>TG</td>
<td>61 (0.328)</td>
<td>37 (0.199)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Allele numbering refers to the SPP1 genomic DNA sequence (GenBank accession no. NT_016354), with the first nucleotide of exon 1 as the transcription initiation start site referred to as nucleotide +1. * Reference SNP in National Center for Biotechnology Information database, June 2006. * Allele counts (allele frequencies). * Allelic frequencies were compared between PXE patients and controls by Fisher 2-sided exact test. * P values <0.05 were considered significant after correction for multiple testing according to the Bonferroni method. P_corrected = P(α) k, k = 6. ND, not determined.

Determination of LD Structure and Haplotype Blocks

LD and haplotype blocks were evaluated in PXE patients and controls using Haploview 3.2 and PHASE v2.1.1 (30, 31). Significant LD was observed among the 6 SPP1 promoter polymorphisms, with a minor allele frequency of >5% as shown in Fig. 1. Determination of LD structure revealed that the 3 variants c.-1748A>G, c.-155_156insG, and c.244_245insTG were in perfect linkage disequilibrium (D’ >0.9), as determined by other groups (24).

The Haploview results showed reconstruction of 8 different haplotype blocks in the pooled sample of cases and controls, but only 5 had a frequency of >2% (Table 3). Comparison of haplotype block frequencies among PXE patients and controls showed a decreased frequency of the major haplotype A (Table 3) in the PXE group (P_corrected = 0.0345, OR 1.8, 95% CI 1.19–2.71). The haplotype showing the highest frequency in the PXE patients (haplotype B in Table 3) carries the alleles c.-1748G, c.-155_156GG, and c.244_245TGTG, which were individually increased in the PXE patients (P_corrected < 0.05 each) (Table 2).

We repeated analysis using the PHASE algorithm to compare haplotype patterns in PXE patients and controls. The PHASE program generated 14 different haplotype blocks (data not shown). The 5 most frequent haplotype patterns and the estimated frequencies were the same as determined by the Haploview algorithm. The remaining 9 haplotypes had frequencies <1%. The overall haplotype frequency distribution was significantly different between patients and controls (P = 0.04).

Association of SPP1 Promoter Polymorphisms with Clinical Features

Allelic frequencies of the 3 SPP1 promoter variants were analyzed in subgroups of the PXE patient groups to evaluate an association with the clinical features shown in Table 1. We found no significant association between age, age at PXE onset, and number or kind of organs involved.

Discussion

PXE is histologically characterized by a progressive calcification and degradation of elastic fibers (1). The implication of a defect or deficiency of MRP6 in the development of PXE, especially in the mineralization process, is still unknown. Characteristic for PXE is a great variability in the clinical course and phenotype of PXE, even among patients with the same or functionally similar ABCC6 mutations. It has been difficult to reveal any association of ABCC6 mutations and a precise PXE phenotype, although
A few studies have reported genotype–phenotype associations in PXE patients (6,15,32). The variability in the outcome and progression of PXE may include variations in functional pathways involved in the pathophysiology of the disease. Any genetic variation in genes regulating these pathways may be involved in the development of PXE. The detection of such genes may uncover major pathways involved in pathogenesis and provide targets for therapeutic intervention.

A number of genes are likely to contribute to PXE susceptibility. Previously we showed that polymorphisms in the xylosyltransferase I and II genes (XYLT1 and XYLTI2) result in a severe disease course of PXE (33). In this study, we analyzed SPP1, an interesting candidate gene.

### Table 3. Estimated SPP1 haplotypes detected in PXE patients and controls.

| Haplotype  | c.−1748 | c.−616 | c.−443 | c.−155_156 | c.−66 | c.244_245 | Overall allele frequencies | Frequency in PXE patients, n = 186 | Frequency in controls, n = 186 | OR (95% CI) | P | P_corrected
|------------|---------|--------|--------|-------------|-------|-----------|--------------------------|-------------------------------|-------------------------------|-----------------|---|----------------
| A A T C G T TG | 183 (0.492) | 78 (0.419) | 105 (0.564) | 1.80 (1.19–2.71) | 0.0069 | 0.0345
| B G T T GG G TGTG | 75 (0.205) | 47 (0.252) | 29 (0.161) | 0.55 (0.33–0.92) | 0.0284 | 0.142
| C A T T G T TG | 67 (0.180) | 28 (0.150) | 39 (0.210) | 1.51 (0.88–2.57) | 0.1399 | 0.6995
| D G G T T GG T TGTG | 16 (0.042) | 12 (0.065) | 3 (0.016) | 0.24 (0.07–0.86) | 0.0316 | 0.158
| E A T C G G T TG | 12 (0.033) | 9 (0.033) | 3 (0.016) | 0.32 (0.09–1.21) | 0.1395 | 0.6975

Haplotypes were determined by use of Haplovie 3.2 software (30). These 5 genotype combinations account for >95% of the total haplotypes detected. The remaining 5% were dispersed in 3 rare genotype combinations found in both PXE patients and controls, with frequencies <2% each. *Allele counts (allele frequencies). **Corrected P value according to the Bonferroni method for multiple testing, k = 5.
gene with important functions in the regulation of biological calcification, for sequence variations in the proximal promoter region. Altered expression of SPP1 has been found in the dermis of PXE patients analyzed by immunoelectron microscopy (19). Recent studies have described strong SPP1 expression due to the Dyscalc1 locus in mice suffering from dystrophic cardiac calcification, with abcc6 gene as a potential candidate gene (20, 21). The abcc6 gene has just been excluded, however, emphasizing that only the N-terminal part of the gene was screened for sequence variations and that most PXE-associated ABCC6 mutations were found in the C-terminal part (5, 6, 34).

The results of our investigation suggest that German PXE patients represent a different distribution of SPP1 promoter polymorphisms than an age- and sex-matched control cohort. The c.–1748A>G, c.−155_156insG, and c.244_245insTG alleles appear to be significantly more common in PXE patients. Haplotype analysis revealed 1 haplotype to be significantly reduced among PXE patients, whereas another haplotype, bearing the disease-associated alleles, was more often found in the PXE group (although not statistically significant). Conclusively, these 3 SPP1 promoter polymorphisms and the haplotype combining these disease-associated alleles could be interpreted as a genetic risk pattern for PXE.

Until now, no functional studies have been carried out with the SPP1 promoter polymorphism c.–1748A>G. The polymorphic variant c.244_245insTG did not have a major effect on regulation of SPP1 gene expression (35). Analysis of the SPP1 promoter sequence revealed putative transcription factor binding sites for SP1 around c.–66, for CBFA1/RUNX2 around c.–155, and for MYT1 zinc-finger factor at c.–443 (Fig. 2) (23). The polymorphism c.–155_156insG generates a RUNX2-binding site. Another RUNX2-binding site was found 14 bp downstream and is highly conserved between species, and the RUNX2 factor was shown to bind better to the c.–155_156GG allele than to the c.–155_156G allele (23). RUNX2-binding sites are very important for regulation of SPP1 expression in bone tissue (36). A constitutive expression of RUNX2, combined with a supplementation of glucocorticoid hormones, resulted in a strong upregulation of SPP1 expres-

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**Fig. 2.** Schematic diagram of the SPP1 promoter region and putative transcription factor binding sites affected by the detected SPP1 polymorphisms.

Sequence variations identified in the present study are indicated. Regulatory motifs affected by the sequence variants c.–443T>C, c.–155_156insG, and c.–66T>G are shown in detail in the lower part of the Fig (23). The affected nucleotides are printed in bold and italics. The putative transcription factor binding sites are labeled by grey lines above or below the sequence.
sion and finally in a biological matrix mineralization of primary dermal fibroblasts (37). Reporter gene expression experiments with the SPP1 promoter polymorphisms c.-443C>T, c.-155_156insG, and c.-66T>G revealed significantly altered expression of SPP1 (23, 25). All haplotype combinations constructed by these 3 sequence variants resulted in increased reporter gene expression. The strongest expression was conferred by the G-insertion in position c.-155 in combination with the c.-66T allele (25). These results suggest that haplotypes should be preferred for disease association studies instead of single variants.

Although we identified polymorphisms and haplotype patterns in the SPP1 promoter that may play a significant role in the pathogenesis of PXE by altering gene expression, the mechanisms by which these variants predispose patients to PXE are unknown. The higher expression of SPP1 found in the dermis of PXE could result from the genetic risk pattern we identified in this study or could just accompany the calcification process. It is still uncertain whether a higher expression of SPP1 prevents or promotes the mineralization process. Phosphoproteins such as SPP1 were shown to initiate mineral formation when bound to collagen and to inhibit crystal growth when in solution (38). Therefore, SPP1 could induce calcification when bound to the scaffold of elastic fibers, as shown by Baccarani-Contri et al. (19). Whether SPP1 is bound to the elastin protein or to another component of the elastic fiber, maybe hyaluronic acid, is still unknown. Thus, the identification of the physiological substrate of MRP6 will shed light on the role of SPP1 and other extracellular matrix proteins in PXE.

There are some limitations to our study. Although the size of the PXE patient cohort was small, the power of the present study was adequate to detect an association of SPP1 promoter polymorphisms and susceptibility to PXE reliably. However, it cannot be totally excluded that relationships of smaller magnitude were missed in our analysis. Especially for the less frequent variants with a minor allele frequency <8%, the power did not exceed 80%, indicating a possibility of false-negative results. Our cohort size was not large enough to detect associations when assuming a dominant or recessive disease model. Another retrospective study analyzing the association of SPP1 promoter polymorphisms in PXE patients is now necessary to determine whether these polymorphisms are indeed a genetic risk factor for PXE.

We have not yet analyzed the effect of these SPP1 promoter variants on the expression of SPP1, for example in plasma or serum, for several reasons. The currently available ELISA assays are suitable for determination of SPP1 concentrations only in plasma samples, which were not available for the PXE patients and controls in this study. Despite several publications that report SPP1 concentrations in serum, the results from currently used ELISA systems should be interpreted with caution because of highly variable and contradictory SPP1 concentrations in plasma and serum samples, even among healthy controls and between identical ELISA systems (39).

In summary, we have observed an association between SPP1 promoter variants and PXE. Our findings add significant support to the role of proteins actively involved in regulating calcification processes in PXE and underscore the importance of the analysis of gene–gene–environment interactions in understanding the development of complex phenotypes such as PXE. Understanding the whole genetic risk pattern or patterns of PXE may provide new insights into the pathogenesis of the disease and eventually provide opportunities for its treatment, which are limited at present (1). For example, regulating the activity of the SPP1 signaling pathway to modulate its effect on calcification may be possible. Further studies are required to analyze the exact role of SPP1 in the pathophysiology of PXE.

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