Concise Report

Investigation of the role of ENPP1 and TNAP genes in chondrocalcinosis

Y. Zhang, M. A. Brown, C. Peach, G. Russell and B. P. Wordsworth

Objectives. Extracellular inorganic pyrophosphate (ePPi) inhibits certain forms of pathological mineralization while promoting others. Three molecules involved in ePPi regulation are important candidates for the development of calcium pyrophosphate dihydrate chondrocalcinosis (CPPD CC). These include ANKH, ectonucleotide pyrophosphatase (ENPP1) and TNAP. We have previously shown that genetic variation in ANKH is a cause of autosomal dominant familial CC and also some sporadic cases of CPPD CC. We now investigate the possible role of ENPP1 and TNAP in CPPD CC.

Methods. Exons, untranslated regions (UTR) and exon–intron boundaries of ENPP1 and TNAP were sequenced using ABI Big Dye chemistry on automated sequencers. Sixteen variants were identified (3 in ENPP1 and 13 in TNAP) and were subsequently genotyped in 128 sporadic Caucasian CPPD CC patients and 600 healthy controls using a combination of polymerase chain reaction/restriction fragment-length polymorphism analysis or using Taqman. Allele and genotype frequencies were compared between cases and controls using the χ² test. Linkage disequilibrium, haplotype and the single nucleotide polymorphism-specific analyses were also performed. This study had 80% power to detect an odds ratio of 2.2 or more at these loci.

Results. No difference was observed in the allele or genotype frequencies between patients and controls at either ENPP1 or TNAP.

Conclusions. Polymorphisms of ENPP1 and TNAP are not major determinants of susceptibility to CC in the population studied. Further studies of the aetiology of sporadic CPPD CC are required to determine its causes.

KEY WORDS: Pyrophosphate arthropathy, ANKH, Pseudogout, ENPP1, TNAP.
activity should also cause reduced ePPi levels and similar phenotypes. ENPP1 knockout mice (Enpp1<sup>−/−</sup>), also known as 'tiptoe walking' (ttw/ttw) mice, spontaneously develop progressive ankylosing intervertebral and peripheral joint hyperostosis and articular cartilage calcification [1, 16]. In humans, variants of ENPP1 have been associated with ossification of the posterior longitudinal ligament (OPLL) [1, 17]. OPLL occurs predominantly in middle-aged and elderly men. It is often asymptomatic but may cause spinal stiffness and occasionally compressive myelopathies [18]. It is also associated with chondrocalcinosis [19].

In human infants, severe ENPP1 deficiency was recently linked to myelopathies [18]. It is also associated with chondrocalcinosis [19]. but may cause spinal stiffness and occasionally compressive myelopathies [18]. It is also associated with chondrocalcinosis [19].

Tissue-non-specific alkaline phosphatase (TNAP), one of a family of four homologous human alkaline phosphatase enzymes, is also present in matrix vesicles. It catalyses the hydrolysis of PPI, thereby decreasing the extracellular concentration of this natural inhibitor of PPI crystallization. The TNAP gene locus on chromosome 1 consists of 12 exons [21]. Deletion of TNAP in mice (Akp2<sup>−/−</sup>) results in a model of infantile hypophosphatasia which is characterised by rickets, osteomalacia, spontaneous bone fractures and increased PPI levels [22–24]. In humans, more than 65 mutations have been reported to cause hypophosphatasia (summarized at http://www.sesep.uvsq.fr/database_hypo/Mutation.html).

In health, the functions of ENPP1 and TNAP appear to be balanced; loss of ENPP1 function causes excess bone formation and mineralization, but loss of TNAP function causes hypomineralization and bone fragility. It is of interest that mice lacking both ENPP1 and TNAP (crossbreeding the Enpp1<sup>−/−</sup> and the Akp2<sup>−/−</sup> mice) have essentially normal ePPi levels [25]. Thus, ENPP1 and TNAP are crucial, directly antagonistic regulators of bone mineralization, influencing the normal steady-state levels of PPI [25, 26].

Recent evidence demonstrates that ANKH variants are linked to at least some familial and some, but not all, sporadic CC [9–11, 27]. Since both ENPP1 and TNAP affect PPI levels, they are strong candidates for involvement in at least some sporadic cases of CPPD CC. We therefore investigated whether variations in ENPP1 and TNAP genes are associated with CPPD CC in a set of Caucasian patients from the UK.

### Patients and methods

#### Patients and controls

All 128 patients with sporadic CC were UK Caucasians. Clinical onset of symptoms occurred after 50 yrs of age and none had a family history of early onset CC. Patients were recruited from out-patient clinics at the Nuffield Orthopaedic Centre in Oxford, UK, following informed consent. The study was approved by the Oxford Research Ethics Committee. Eighty patients (62.5%) were ascertained by radiographic evidence of chondrocalcinosis (defined as widespread disease involving linear cartilage calcification of at least two knee compartments and/or involvement of or more than two joints other than the knee); 48 patients (37.5%) were identified from the records of synovial fluid analysis in the histopathology department. Patients with known hypercalcaemia or haemochromatosis were excluded. Six hundred healthy Caucasian blood donors from the same geographic area were studied as controls. Genomic DNA was extracted from peripheral blood leucocytes from all the study subjects by chloroform/phenol methods.

**Mutation screening and genotyping**

Mutation screening was performed by direct sequencing of the 25 exons of ENPP1 and 12 exons of TNAP, including the exon-intron boundaries after polymerase chain reaction (PCR) amplification (primer pairs listed in Supplementary data 1). DNA from 24 patients with CC and 24 from healthy controls was sequenced. Using 48 samples, there was 95% power to detect minor alleles with frequency of 0.03. Sequencing was carried out using ABI Big Dye chemistry on an ABI 3700 automated sequencer (Applied Biosystems, UK). The single nucleotide polymorphisms (SNPs) were subsequently genotyped either by PCR/restriction fragment-length polymorphism analysis (RFLP) or by Taqman (Kbiosciences, UK).

**Statistical analyses**

ENPP1 and TNAP genotype and allele frequencies in patients and controls were compared by standard x² contingency table analysis. Genotype relative risks were estimated by the method of Lathrop [28].

**Linkage disequilibrium analyses**

The pair-wise linkage disequilibrium (LD) (D') between SNPs was calculated by haploxt and summarized graphically by the program Graphical Overview of Linkage Disequilibrium (GOLD) [29].

**Haplotype and the SNP-specific analyses**

Single marker and two-marker sliding window haplotype analyses were also performed using the program ‘Whap’ (available at URL: http://www.genome.wi.mit.edu/~shaun/whap). Empirical significance levels were determined by permutation testing, and are corrected for the number of analyses performed, taking into account LD between markers.

**Power calculation**

This study has 80% power to detect an association at a significance level (P = 0.05) with an odds ratio of 2.2 or more, assuming a codominant genetic model, and that the genotyped SNPs themselves are disease-associated variants or are in complete linkage disequilibrium (LD).

### Results

Three variants were identified in ENPP1 and 13 variants in TNAP. None of these variants has been reported in Hap Map. There was one non-synonymous SNP in ENPP1, but three out of four coding region SNPs in TNAP changed amino acids (Table 1). Two SNPs in ENPP1 and six in TNAP were novel. Genotypes of all the SNPs of ENPP1 and TNAP were in Hardy–Weinberg equilibrium. No association was detected between any of the SNPs and CC (Table 1). Empirical P-values from 100 permutations for ENPP1 were >0.1 and for TNAP were >0.3.

LD in the region of the first SNP to the third SNP in ENPP1 was estimated, giving values for D' of 0.93, 0.33 and 0.45 (1 = full LD, 0 = no LD). For TNAP, D' was greater than 0.88 between most SNP (Graphic representation of the LD patterns shown in Supplementary data 2).

### Discussion

In this study, eight new SNPs in ENPP1 and TNAP were identified. We have provided strong evidence that ENPP1 and TNAP are not significantly involved in sporadic CPPD CC of late onset. This study had good power (80%) to detect the relatively strong genetic effects (equating to odds ratio of 2.2 or more) that might be found in complex traits with polygenic influences. Three and thirteen polymorphisms in the coding region and exonic flanking sites were identified in ENPP1 and TNAP, respectively, and were further genotyped. No association of any
ENPP1 or TNAP variants with susceptibility to disease was found. Although one coding region SNP in ENPP1 and three out of four coding region SNPs in TNAP changed amino acids (Table 1), these were all conservative substitutions (hydrophilic amino acids). However, because of the power of the analysis, it is not possible for us to exclude modest genetic effects of the variants in this study.

ENPP1 and TNAP have contrasting influences on ePPi aberrant levels of which are strongly associated with CC. However, our results strongly suggest that variants of these genes are unlikely to be a major cause of CPPD. Two of the three SNPs in ENPP1 are in strong LD while all the variations in TNAP show very high LD; it is therefore highly unlikely that any other common polymorphisms in the unsequenced region of introns or regulatory sequences causing CC exist in either gene.

Although ENPP1 polymorphisms have been associated with OPLL and juvenile vascular calcification [1, 30], a firm causal relationship is yet to be established [12]. Suk and colleagues [31] recently reported a short tandem repeat polymorphism located in the promoter region of ENPP1 that is associated with hand osteoarthritis (OA) in an isolated Chuvashian population. A high prevalence of CPPD crystals in OA joints has also been reported [32], but whether there is any common polymorphism in the unsequenced region of ENPs resulting in calcium pyrophosphate deposition in English families. Br J Rheumatol 1991;30:10–5.}

### Table 1. ENPP1 and TNAP genotype frequencies in patients with CC and healthy controls

<table>
<thead>
<tr>
<th>Exon</th>
<th>Position(^b) (bp)</th>
<th>Nucleotide variation/aa change(^d)</th>
<th>ENPP1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>CC patients</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.1 (%)</td>
</tr>
<tr>
<td>Exon 2</td>
<td>+10(^c)</td>
<td>g/t</td>
<td>91 (84)</td>
</tr>
<tr>
<td>Exon 4</td>
<td>87</td>
<td>A/C (Lys/Glu)</td>
<td>76 (70)</td>
</tr>
<tr>
<td>Exon 21</td>
<td>−14(^c)</td>
<td>t deletion</td>
<td>67 (60)</td>
</tr>
<tr>
<td>Exon 3</td>
<td>+52</td>
<td>c/a</td>
<td>41 (38)</td>
</tr>
<tr>
<td>Exon 5</td>
<td>33</td>
<td>C/T (Ser/Thr)</td>
<td>91 (83)</td>
</tr>
<tr>
<td>Exon 7</td>
<td>139</td>
<td>T/C (Tyr/His)</td>
<td>106 (97)</td>
</tr>
<tr>
<td>Exon 8</td>
<td>+45</td>
<td>g/t</td>
<td>93 (90)</td>
</tr>
<tr>
<td>Exon 9</td>
<td>−46(^c)</td>
<td>g/a</td>
<td>88 (82)</td>
</tr>
<tr>
<td>Exon 10</td>
<td>−12(^c)</td>
<td>c/g</td>
<td>83 (81)</td>
</tr>
<tr>
<td>Exon 14</td>
<td>14(^c)</td>
<td>A/G (Glu/Arg)</td>
<td>90 (83)</td>
</tr>
<tr>
<td>3’-UTR</td>
<td>+65(^c)</td>
<td>c/t</td>
<td>35 (35)</td>
</tr>
</tbody>
</table>

\(^a\)Individual alleles are recorded with reference to those published in public databases, where allele 1 is the common wild type and allele 2 the less common variant.  
\(^b\)SNPs are listed with reference to their positions either in front ‘−’ of the first nucleotide or after ‘+’ the last nucleotide of each exon.  
\(^c\)SNP not previously reported.  
\(^d\)Intronic variations shown in lower case; exonic variations in upper case; aa, amino acid.  
\(^e\)After the stop codon.

In conclusion, our mutation screening and genotyping results indicate that variants of ENPP1 and TNAP are unlikely to provide a major genetic contribution to sporadic forms of CPPD in our CC population. However, we cannot formally exclude a role for these genes in other ethnic groups.

### Acknowledgments

We are grateful to Prof. N. Athanasou for providing the results of synovial fluid crystal analysis. This work was funded by the Arthritis Research Campaign and the Royal College of Surgeons of England.

The authors have declared no conflicts of interest.

### References

Chondrocalcinosis and ENPP1 and TNAP genes