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Mutations in the Amino Terminus of ANKH in Two US Families With Calcium Pyrophosphate Dihydrate Crystal Deposition Disease

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Objective. To analyze ANKH in families with calcium pyrophosphate dihydrate crystal deposition disease (CPPD) for disease-causing mutations.

Methods. Two US families (one of British ancestry and the other of German/Swiss ancestry) with autosomal-dominant CPPD, whose disease phenotypes were found to be linked to chromosome 5p15.1 (locus symbol CCAL2), were screened by direct sequencing for mutations in ANKH, a gene in the CCAL2 candidate interval that has been shown to harbor mutations in other families with CPPD. Observed sequence variants were confirmed by antisense sequencing, and expression of the mutant allele was verified by reverse transcriptase–polymerase chain reaction amplification of messenger RNA followed by direct sequencing.

Results. The two US families displayed the same mutation at position 5 of the ANKH gene product (P5T). All affected members were heterozygous for the P-to-T variant, and the mutation was not seen in 204 control alleles. The two families displayed distinct disease haplotypes, suggesting that they were unrelated to each other.

Conclusion. These observations represent the fourth and fifth families with heritable CPPD whose disease phenotypes are linked to the CCAL2 locus and who have missense mutations in the amino terminus of ANKH. This same position (P5) was the site of a missense mutation in an Argentinean family of northern Italian ancestry; however, the sequence variant in that family generated a P5L mutation. The distinct disease haplotypes among the 3 families with P5 mutations suggest that the mutations arose independently and that the evolutionarily conserved P5 position of ANKH may represent a hot spot for mutation in families with autosomal-dominant CPPD.

Calcium pyrophosphate dihydrate crystal deposition disease (CPPD) is a disease of articular cartilage that is radiographically characterized by the deposition of calcium-containing crystals in the joint (1,2). When it occurs as a familial disorder, it is usually inherited in an autosomal-dominant manner, and affected family members may display symptoms of disease as early as their third decade of life. Several families with multiple generations of affected members have been studied by parametric genetic linkage analysis, and a locus on the short arm of chromosome 5 (locus symbol CCAL2, MIM 118600) has been linked to the disorder (3,4).

Ho et al (5) described the cloning of a gene that was responsible for a recessive phenotype in the *ank* (progressive ankylosis) mouse. The human homolog for this gene, ANKH (MIM 605145), was located in a region syntenic with the mouse chromosomal assignment on human chromosome 5p. Because of its location in the CCAL2 candidate interval and its reported function as a regulator of inorganic pyrophosphate (PPi) transport (5), ANKH became an obvious positional candidate

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gene for CPPD. Mutation analyses of ANKH in the 3 families for which linkage analyses were performed (3,4) discovered 3 distinct mutations at the amino terminus of the coding region for the gene. In a British family with CPPD, a base substitution just upstream of the translation start codon introduced a new translation initiation point that, in *in vitro* analysis, resulted in the translation of a protein from the mutant allele that was 4 amino acids longer than that translated from the normal allele (6). In a French family with CPPD originally described by Gaucher et al (7), a missense mutation in the second exon of the ANKH gene resulted in an M48T heterozygous sequence variant in all affected family members (6). Finally, in an Argentinean family of northern Italian descent, a base substitution in the first exon of the gene resulted in a P5L missense mutation in affected members (8).

In the present study, we observed a new missense mutation at amino acid position 5 of the ANKH gene product. In two US families of British and German/Swiss ancestry, the missense mutation was a transversion that changed the first basepair of the proline codon (CCG) to a threonine (ACG).

PATIENTS AND METHODS

Family studies and isolation of genomic DNA and total RNA. The two US families with radiographically confirmed CPPD consisted of 9 affected members and 15 unaffected members from 3 generations. In family US-A, the proband, member III:8, had had numerous episodes of pseudogout since her third decade of life; this was also the case for other affected members of this kindred, including members II:6, III:2, III:3, and III:12. All affected members also reported having chronic joint symptoms and developed osteoarthritis later in life. Radiographic evidence of chondrocalcinosis demonstrated involvement of both fibro- and hyaline cartilage and mainly involved the knees and feet. There was no evidence of extraarticular calcification. Calcium pyrophosphate dihydrate crystals were microscopically identified in joint effusions from affected members.

Details concerning the phenotype of family US-B were previously described (9). Affected members of this family had radiographically confirmed degenerative joint disease, and about one-half of the affected members had acute episodes of arthritis superimposed on their chronic joint symptoms. Most affected members demonstrated polyarticular disease with involvement of both fibro- and hyaline cartilage. In rare cases, patients in the family were asymptomatic despite having radiographic evidence of chondrocalcinosis. This family was also examined for pyrophosphohydrolase activity and PPi content in cultured skin fibroblasts of some affected members (10).

For genotype analyses and mutation screening, genomic DNA was extracted from peripheral blood samples obtained from family members using the PureGene DNA Isolation Kit (Gentra Systems, Minneapolis, MN). The project

to define the molecular basis for familial chondrocalcinosis was approved by the Institutional Review Board at Thomas Jefferson University. For reverse transcriptase-polymerase chain reaction (RT-PCR) analyses, total RNA was extracted from peripheral blood leukocytes using the QIAamp RNA Blood Mini Kit (Qiagen, Valencia, CA).

Linkage analyses. Genotyping of family members using fluorescently labeled polymorphic markers from the CCAL2 region (D5S817, D5S1991, D5S1954, D5S1963, D5S416, D5S1997) was performed, and PCR products were visualized on an ABI 373 sequencer and analyzed with GeneScan software (Applied Biosystems, Foster City, CA). Two-point linkage analyses were performed with the FASTLINK versions of the LINKAGE package of programs as previously described (4) under an autosomal-dominant mode of inheritance. Haplotypes for family members were manually derived from inspection of genotype data.

Sequence analysis of ANKH. All 12 exons of the ANKH gene were sequenced from genomic DNA of probands from each family and several unaffected and 102 unrelated, racially matched controls. Primers for amplification by the PCR were designed to include at least 100 bp of flanking intronic sequence. Sequencing was performed by automated dideoxynucleotide terminator chemistry on an ABI 377 sequencer. Some PCR products were subcloned using the TA cloning vector system (Invitrogen, Carlsbad, CA), and clones containing inserts were subjected to DNA sequencing using vector sequences as primers. Sequence variants were confirmed by sequencing the products with putative variants with the antisense primer.

The primers for the amplification of genomic DNA for exon 1 that harbored the sequence variants were as follows: ANK -248 (sense), 5'-GCAGATCTTTGTTGTGTGGG-3'; ANK 85C (antisense), 5'-AGTCGATGGCTATGTTGGTG-3'. For improved resolution of PCR products, some reactions were subjected to a second round of PCR using a nested antisense primer: ANK 55C, 5'-GCACCAAGAACCGGATCAGG-3'. The primer numbers 85C and 55C denote the positions of the primers on the complementary (C) strand of exon 1; the -248 sense primer refers to the position of this primer in the 5'-untranslated region (5'-UTR) (where position 1 is A of the ATG initiation codon). PCR reactions were performed for 35 cycles using an annealing temperature of 60°C and including 5% DMSO as an adjunct to aid in denaturation of the GC-rich region that resides 5' of the initiation codon. The outside and nested primer pairs generated PCR products of 334 bp and 304 bp, respectively.

For RT-PCR analyses, RNA was used as template for the generation of complementary DNA (cDNA) and was subjected to the PCR using the ThermoScript RT-PCR system (Invitrogen) using the following primers: ANK -37 (sense), 5'-CAGCAGATGTGTGTGGGGTCAG-3'; ANK 497C (antisense), 5'-GATTGAGGCACATCCCACCAGG-3'; ANK 414C antisense (nested), 5'-GAAAGCGGCGAGGTA-CAGGAA-3'. The primer numbers 497C and 414C denote the cDNA positions of the primers on the C strand of exons 4 and 3, respectively; the -37 sense primer refers to the position of this primer in the 5'-UTR (where position 1 of the cDNA is A of the ATG initiation codon). PCR amplifications of reverse-transcribed cDNA were performed for 35 cycles under standard conditions at an annealing temperature of 58°C. The

outside and nested primer pairs generated PCR products of 535 bp and 452 bp, respectively.

RESULTS

The pedigrees of both US families are depicted in Figure 1. Genetic linkage analyses and haplotype reconstructions were performed on both families and these analyses confirmed that the disease phenotypes in the families were linked to the CCAL2 locus on chromosome 5p15.1 (data not shown).

In light of previous observations of mutations in

Figure 1. Pedigrees of the two US families described in the present report. All individuals shown were screened for mutations in ANKH. Closed symbols represent individuals affected with calcium pyrophosphate dihydrate crystal deposition disease (CPPD). Open symbols represent unaffected individuals. Arrows indicate probands. Members IV:1 and IV:3 of family US-A and member III:1 of family US-B carry the disease haplotype and the P5T mutation in ANKH, but were too young at the time of DNA sample collection to display the CPPD phenotype.

ANKH in families with CPPD whose disease phenotypes were linked to the 5p15.1 chromosomal interval (6,8), ANKH was screened for disease-causing mutations in genomic DNA from affected and unaffected members of each family by direct sequence analysis. Several neutral polymorphisms were observed that included a third base variant at amino acid position 98 (GCT→GCC) and a base substitution in IVS10 at position +33 with respect to the end of exon 10 in family US-A. However, an amino acid change in exon 1 was noted in both families. This change was generated from a point mutation in one allele of the exon to change a proline (CCG) to a threonine (ACG) (Figure 2A). The presence of the mutation was confirmed by antisense sequencing of the genomic DNA PCR product (data not shown), and its presence in mature transcripts of ANKH was confirmed by RT-PCR analysis of messenger RNA isolated from peripheral blood lymphocytes of 2 affected members of family US-A. Figure 2B illustrates antisense sequencing of cloned mutant and normal transcript alleles from member III:8 of family US-A.

Table 1 lists the mutations in ANKH that have now been described in families with CPPD whose disease phenotypes have been linked to chromosome 5p15.1. The reported mutations are clustered in the amino terminus of the coding region of ANKH.

DISCUSSION

Although the deposition of calcium-containing crystals in articular cartilage (i.e., chondrocalcinosis) is a common finding that is frequently associated with advanced osteoarthritis, the familial manifestation of this disorder is relatively rare. In its most severe form, the disorder may occur as an acute-onset disease resulting in considerable pain and disability. Ultimately, patients display significant degenerative joint disease.

The most commonly observed crystals in familial chondrocalcinosis are calcium pyrophosphate dihydrate crystals, and familial calcium pyrophosphate dihydrate deposition has been linked to loci on chromosomes 8q (locus symbol CCAL1, MIM 600668; ref. 11) and 5p (locus symbol CCAL2, MIM 118600; refs. 3 and 4). Among the positional candidates in the 5p locus was the human homolog of a mouse mutation called progressive ankylosis (*ank*). Recessive mutant mice (*ank/ank*) display calcification of numerous joints (in the form of deposition of hydroxyapatite) and arthritis (12,13). The *ank* gene codes for a protein with putative multipass transmembrane domains, and this protein may function

Figure 2. A, Genomic DNA sequence from polymerase chain reaction (PCR) analysis of exon 1 from one unaffected family member and from one affected family member of kindred US-A. B, Reverse transcriptase (RT)-PCR analysis of an affected member of kindred US-A. RT-PCR products were cloned, and the antisense sequence is depicted.

as an ion channel for the transport of PPi across the cell membrane (5). Although familial CPPD demonstrates a crystal type and other phenotypic features that are somewhat different from those of the mouse mutant, heterozygous

Table 1. ANKH mutations in families with calcium pyrophosphate dihydrate crystal deposition disease whose disease phenotypes have been linked to chromosome 5p15.1*

Family	Exon	cDNA position†	Amino acid position‡	Type of mutation	Ref.
British	5'-UTR	-11	NA	Base substitution (INS+4)	6
French	2	143	48	Missense	6
Argentinean	1	14	5	Missense	8
US-A	1	13	5	Missense	Present study
US-B	1	13	5	Missense	Present study

* cDNA = complementary DNA; 5'-UTR = 5'-untranslated region; NA = not applicable; INS+4 = insertion of 4 amino acids.
† With respect to A of the ATG initiation codon equal to cDNA position 1.
‡ With respect to methionine at this position equal to amino acid 1.

mutations in ANKH have now been observed in several families with CPPD, including the two new families described here. The mutations that we have described are in contrast to that reported for an Argentinean family of northern Italian ancestry, in which the second position of the P5 codon (CCG) underwent a transition mutation to generate a leucine (CTG; ref. 8). Both US families displayed unique haplotypes in the region of chromosome 5p that harbors the ANKH gene (data not shown); these haplotypes were distinct from that observed for the Argentinean kindred. Taken together, these observations suggest that the P5 mutations in all 3 families arose independently, and that this position in ANKH may be a hot spot for mutation.

The decisive proof that a mutation in a gene gives rise to the genetic disorder under analysis must reside at the level of alteration of protein function. Previous in vitro functional studies of the mouse nonsense mutation (a G-to-T transversion that substitutes a stop codon for a glutamine) by Ho et al (5) demonstrated that expression of that recessive mutation results in a significant increase in intracellular PPi levels and a concomitant reduction in extracellular PPi. Since PPi is a potent

inhibitor of hydroxyapatite deposition, it has been hypothesized that a decrease in extracellular PPI (loss of function) might promote crystal deposition in the joints of the mutant *ank/ank* mouse (5). Interestingly, similar in vitro functional analyses of two of the human mutations did not significantly affect intracellular levels of PPI (6). Although measurements of extracellular PPI levels were not performed, it was hypothesized that the human mutations were likely to result in small increases in extracellular PPI levels (gain of function) (6), consistent with proposed models of calcium pyrophosphate dihydrate crystal deposition resulting from increased extracellular levels of PPI (for review, see ref. 14).

Future analyses of the ANKH mutations described here and elsewhere in both in vitro and in vivo systems will enable the elucidation of derangement in the function of ANKH that leads to abnormal PPI metabolism resulting in the pathologic deposition of calcium pyrophosphate dihydrate crystals in the joints of affected family members. Ultimately, the extension of these studies to forms of chondrocalcinosis that display nongenetic etiologies may assist in identifying and developing effective therapeutic modalities for many more patients.

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