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Cathepsin K gene mutations and 1q21 haplotypes in patients with pycnodysostosis in an outbred population

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The molecular genetics of the autosomal recessive disorder pycnodysostosis was studied in five independent families from an outbred Caucasian population. We found two new mutations and one recently described mutation in the cathepsin K gene by sequencing DNA from eight patients with pycnodysostosis: a one base transition in exon 8, c926T > C, causing a single amino acid substitution leucine ☐ proline, L309P; A 3' splice site mutation in intron 2, c121–1G > A, causing deletion of all exon 3, 41V–81Mdel; and the exon 3 missense mutation c236G > A leading to residue G79E. In three of the families patients were homozygous for 926T > C. In the remaining two families patients were heterozygous for 926T > C and 121–1G > A in one case, and for 926T > C and 236G > A in the other case. Assays using genomic DNA were developed for all three mutations. We tested 150 healthy control persons and observed the mutation frequencies: 0 to 300 for 121–1G > A and 236G > A and 1 to 150 for 926T > C. One patient from each family was haplotyped with eight microsatellite markers surrounding the cathepsin K gene on chromosome 1q21. A very rare, $P = 1.8 \times 10^{-6}$ to $P = 0.0004$, and highly preserved area around the presumed disease locus was common to all the patients. This haplotype was found on seven chromosomes identical by state, IBS, out of the possible eight carrying the 926T > C mutation. Founder effect, locus homogeneity, and allele heterogeneity regarding pycnodysostosis within this population are discussed. Finally, the first pregnancy and delivery described in a patient with pycnodysostosis is reported. European Journal of Human Genetics (2000) 8, 431–436.

Keywords: pycnodysostosis; cathepsin K (CTSK) gene; mutation; haplotype; founder effect

Introduction

Pycnodysostosis (MIM No. 265800) is a rare autosomal recessive disorder characterised by short stature, delayed closure of the cranial sutures, loss of the mandibular angle, dysplastic terminal phalanges of the hands and feet, and increased bone fragility. The disorder was first described in 1962.[1,2]

The gene for pycnodysostosis has been mapped to chromosome 1q21 independently by two groups[3,4] to a 6 and 4 cM interval, respectively, and later narrowed down to a 2 cM region.[5] Mutations in the cathepsin K gene (NCBI Unigene No. U13665), a cystein protease gene highly expressed in osteoclasts, have been found to be responsible for pycnodysostosis. Three intragenic mutations were reported[6,7] in three large inbred families with Moroccan Arab, American Hispanic and Israeli Arab ethnic backgrounds, respectively. Three mutations were found in two non-consanguineous families of different ethnic origin.[8,9] Another seven mutations were described in nine unrelated families.[10,11]

To focus on the question of locus heterogeneity and allele heterogeneity within the same population we here present a study of mutations and haplotypes in eight patients from five independent families with pycnodysostosis in an outbred Caucasian population.

Material and methods

Patients

A total of nine patients with pycnodysostosis was admitted to either the paediatric or the orthopaedic department at Herning Central Hospital over a period of 46 years mainly due to neonatal respiratory problems, retarded growth, dysmorphic features, or frequent and complicated bone fractures. Eight of the patients and six of their parents wished to participate in the study (Figure 1). The age of the patients ranged from 3 to 50 years. The control panels consisted of 50 healthy Danish females (panel A) and 100 healthy Danish adults, sex ratio 0.5, (panel B), respectively. The size of the population in the county served by Herning Central Hospital was 271 855. All the controls were collected in the western part of Denmark from a total population of 2.9 million.

The local ethical committee has approved the project, and the participants have given informed consent.

After we ended the study one of the pycnodysostosis patient (II:9) gave birth to a healthy girl born after an uncomplicated pregnancy. An elective Caesarean was performed three weeks prior to the estimated due day, to prevent complications during labour. The birth weight was 2500 g.

Neither the healthy father nor his daughter have yet been tested for pycnodysostosis haplotypes or mutations. Pregnancy and delivery among patients with pycnodysostosis have not previously been described in the literature.

DNA sequencing

Total RNA was made from EBV transformed lymphocyte cultures. For cDNA synthesis 200 ng RNA was used in the Titan™ One Tube RT-PCR System (Boehringer Mannheim, http://biochem.boehringer-mannheim.com) in a total volume of 50 μl. The primers were the external set of primers used by Gelb *et al.*[6] Then nested priming was performed with 1 μl RT-PCR product in a total PCR mix of 50 μl using the internal primer set[6] to amplify a 1129 bp fragment of the cathepsin K gene. After using High Pure PCR Product Purification Kit (Boehringer Mannheim, http://biochem .boehringer-mannheim.com) the internal set of primers was used again for the first cycle sequence preparation (ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit, Perkin-Elmer). The samples were precipitated with ethanol and sodium acetate. Sequence analysis with a ABI Prism 377 (Perkin-Elmer, Langen, Germany) was successful only in the antisense direction. This might be explained by divergence of the forward primer in the two terminal base pairs from the wildtype base sequence (NCBI Unigene accession U13665). The correct nested sense primer should be 5'-CCCCTGATGGTGTGCCCAC-3'. To overcome this problem we designed another set of internal primers for a second
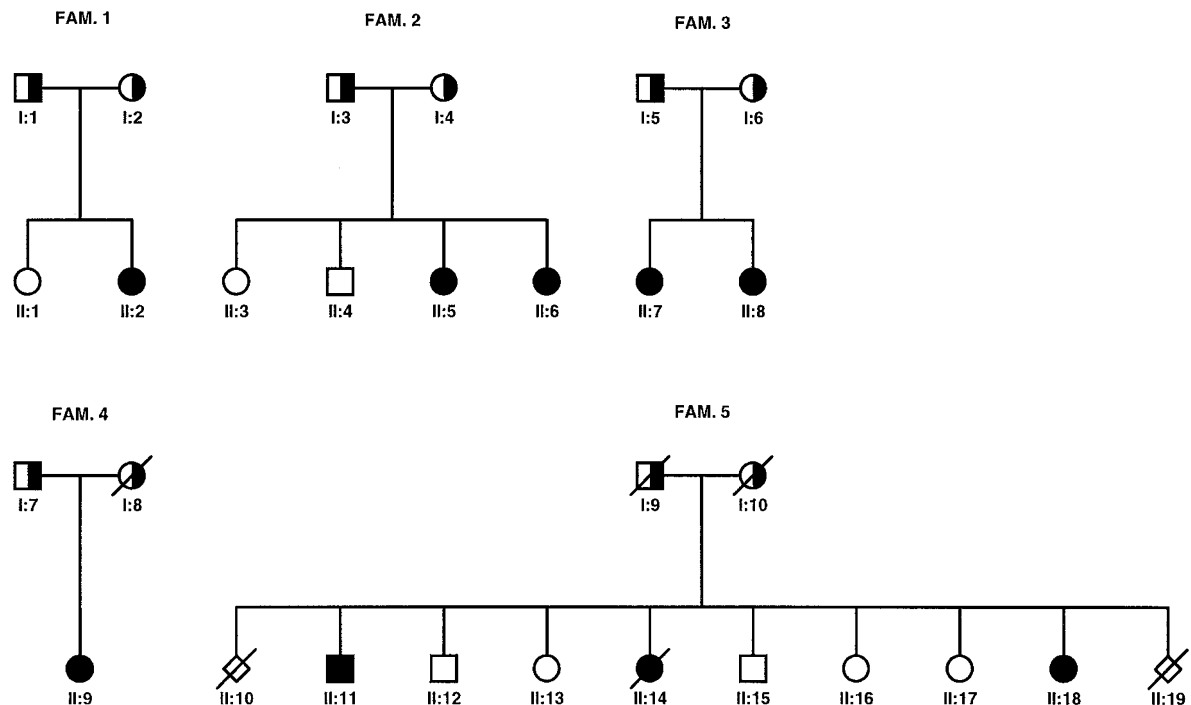

Figure 1 Pedigrees of the five pycnodysostosis families.

sequence analysis of the cDNA. The primers were 5'-GAAAG-GATATGTTACTCCTG-3' (sense) and 5'-AGCCCAACAG-GAACCACACT-3' (antisense).

Mutation analysis

The 121–1G > A mutation assay was based on a 187 bp PCR fragment from amplification of gDNA with the primers: 5'-CCTTTATGATTGTGAGTTTCC-3' (sense) located in intron 2 and 5'-TGTATGGACACCAAGAGAAG-3' (antisense) located in exon 3. The amplifications were performed in a Biometra Personal Cycler and 9 ng DNA was used in a total PCR mix of 6 μl. The PCR conditions were: 1 cycle of 94°C for 5 min, 55°C for 30 s, and 72°C for 36 s; 26 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 36 s; 1 cycle of 94°C for 30 s, 55°C for 30 s, and 72°C for 10 min. Fragments of 119 bp , 94 bp, 68 bp, and 25 bp were separated in a 4% NuSieve gel after incubating 10 μl PCR product with 5 units *Tru*9I restriction enzyme overnight at 37°C. In this assay the 68 bp fragment was common to all the individuals due to a second and constant *Tru*9I restriction site (Figure 3).

The 236G > A mutation was tested by amplifying cDNA with the primers 5'-TGGGAGCTATGGAAGAAGA-3' (sense) and 5'-GTAACATATCCTTTCTTTCG-3' (antisense). Annealing temperature was 51°C and the remaining PCR conditions as described above. A 308 bp PCR fragment was digested with *Mnl*I for 12 h at 37°C. Gel electrophoreses showed bands of 138, 104, 66 and 49 bp. Amplification of gDNA gave a 857 bp PCR fragment and a mutation band of approximately 100 bp (Figure 3). The accurate band length could not be determined due to unknown restriction sites in introns 2 and 3.

To test for the 926 > C mutation a PCR fragment of 523 bp was amplified from gDNA using the primers 5'-CTGGGGA-GAAAACTGGGGAA-3' (sense) and 5'-CCTTGAGGATATT-GAAGGGAACTTAG-3' (antisense). The latter was identical to the first reverse primer used in the nested priming. The PCR conditions were as earlier described. After incubating 10 μl PCR product overnight at 37°C with 5 unit restriction enzyme *Mnl*I the fragments (523 bp, 480 bp and 43 bp) were separated in a 4% NuSieve gel (Figure 3). An extra *Mnl*I restriction site was expected in the 523 bp fragment from viewing the wildtype sequence, but sequencing the area showed a divergence from the database in about position 1171 where the nucleotide was A instead of T.

Haplotyping

DNA was extracted from 10 ml EDTA peripheral blood by the simple salting out procedure.[12,18] In total eight microsatellite markers were labelled radioactive with [33P]-γATP. The primer sequences and the genetic distances were obtained from GDB (http://gdbwww.gdb.org/) and from Marshfield (http://www.marshmed.org/genetics/), respectively. PCR conditions were as described under the mutation analysis. For D1S303 the annealing temperature was 60°C. Formamide-based dye was added followed by denaturation at 94°C for 5 min. Electrophoresis was performed in a 6% polyacrylamide gel with 7 M urea and run for 2 h at 60 W. The gel was dried and exposed to X-ray films for 1–5 days. Two persons scored the marker alleles independently. The estimated haplotype frequencies in the population were calculated by multiplying the individual marker allele frequencies obtained from the CEPH database (http://www.cephb.fr/).

Results

Eight pycnodysostosis patients from five Danish families containing three sib pairs with the disease were included. A full medical and family history was obtained from each patient. Clinical photos and X-rays of the cranium, mandibular angle and left hand were taken to document the diagnosis. Blood for chromosomal analysis, DNA extraction and lymphocyte culturing were sampled. The eight patients
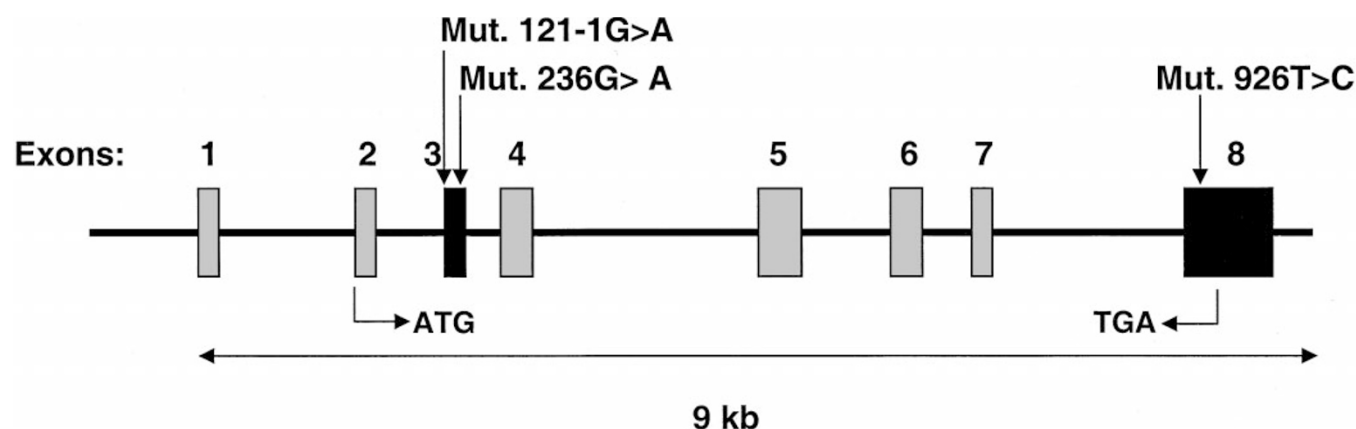

Figure 2 Genomic structure of the human cathepsin K gene (12) showing the localisation of the three mutations and 121–1G > A, 236G > A and 926T > C.
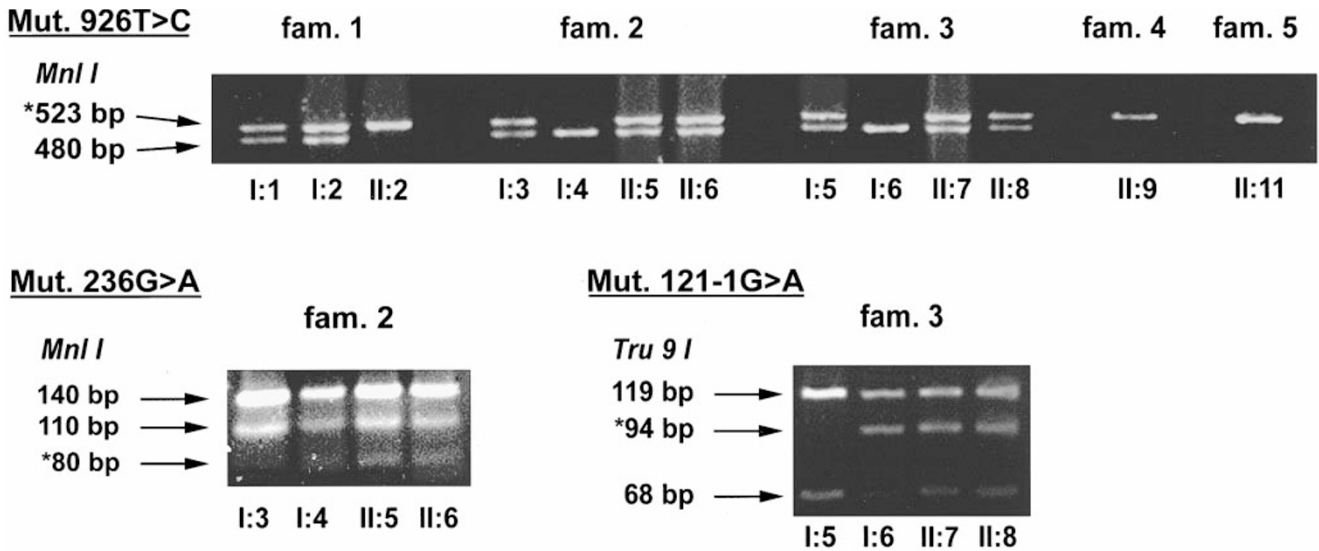
Figure 3 The three RFLP mutation assays showing the use of the restriction enzymes (*Mnl*I, *Tru*9I), fragment length in base pair (bp) and segregation of the disease alleles* in the families (1–5). The fragment lengths of mutation assay 236G > A are approximate. Genomic DNA was amplified.

represented five nuclear families (Figure 1), that could not be related four generations back. The pedigrees revealed no consanguinity. Three of the patients have previously been described clinically, patients no. II:7, II:11 and II:18.[13] No chromosomal abnormalities were detected in the patients.

EBV transformed lymphocyte cultures were obtained from 5 patients (II:2, II:7, II:8, II:9, and II:11) and cathepsin K gene cDNA was made from mRNA. Sequencing the gene in the five patients revealed three different mutations:

1. a major deletion of 123 bp from cDNA nucleotide position 121 to 243 predicting a loss of 41 amino acids in the cathepsin K polypeptide, 41V–81Mdel;

2. a one base G □ A transition in cDNA nucleotide position 236 changing a glycine to a glutamine residue, G79E;

3. a one base T □ C transition in cDNA nucleotide position 926 predicting a substitution of a leucine to a proline at residue 309 in the mature peptide, L309P.

The 236G > A mutation was described recently.[10,11] By viewing the described structure of the human cathepsin K gene[14] the 123 bp deletion turned out to be a full deletion of exon 3, and the one base substitution at about 926T > C occurred in exon 8 within the coding region (Figure 2). The exon 3 deletion was further investigated by PCR amplification from genomic DNA of a 925 bp fragment covering all exon 3 and intron 2 and 3. Sequencing showed a splice site mutation in the last nucleotide position in intron 2, about 121–1G > A, to be responsible for the total exon 3 skipping. Predictions of the secondary protein structure by an IBCP-Web server analysis[15,16] showed the 926T > C mutation to result in limitation of a beta-sheet domain and the

121–1G > A mutation to alter a large domain of both coils and helixes to a small domain of only coils.

RFLP based assays were designed for all three mutations, and patients and parents were tested. This confirmed segregation of the mutations with the disease in the families (Figure 3). The three mutations were tested in two independent panels of healthy adults from the Danish population – consisting of 50 (panel A) and 100 (panel B) persons, respectively. Two persons from panel A turned out to be heterozygous for 926T > C, whilst none from panel B carried the mutation giving a frequency of 1:150. The two heterozygous controls were also sequenced with respect to exon 8, which confirmed that they did carry the 926T > C mutation. Neither 121–1G > A nor 236G > A was found in any of the 150 controls.

We used eight Genéthon microsatellite markers from chromosome 1q21 surrounding the cathepsin K gene to determine the haplotypes (Figure 4). The parents were haplotyped so as to recognise the phase when necessary and possible. Three out of eight chromosomes carrying the mutation 926T > C also carried an extended haplotype, a *master haplotype*, of approximately 9 cM. Assuming linkage equilibrium the frequency of the master haplotype was estimated, $P = 1.8 \times 10^{-6}$. Seven out of eight chromosomes carried preserved pieces of varying length of the master haplotype. The longest piece of haplotype common to all seven chromosomes, the *core haplotype*, was characterised by markers D1S442, D1S498 and D1S2345, $P = 0.004$. Observing this rare core haplotype seven out of eight times strongly indicated a founder effect. One chromosome in patient II:2 did not have the core haplotype despite carrying the common mutation 926T > C.
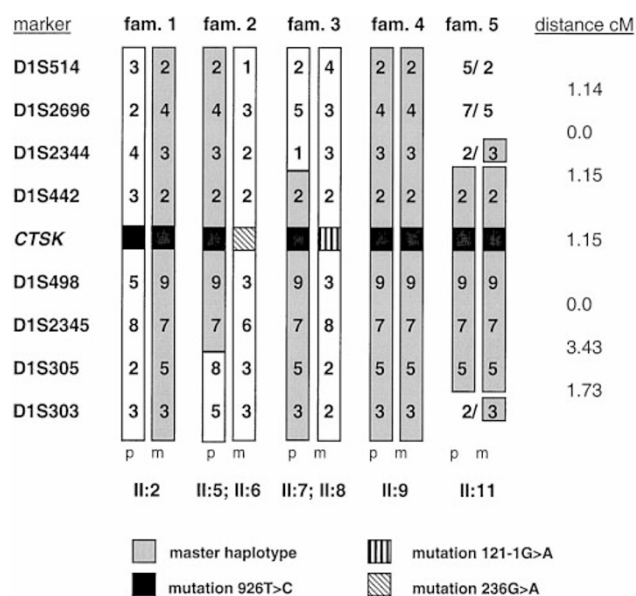
marker	fam. 1		fam. 2		fam. 3		fam. 4		fam. 5		distance cM
D1S514	3	2	2	1	2	4	2	2	5/ 2		1.14
D1S2696	2	4	4	3	5	3	4	4	7/ 5		0.0
D1S2344	4	3	3	2	1	3	3	3	2/ 3		1.15
D1S442	3	2	2	2	2	2	2	2	2	2	1.15
CTSK											1.15
D1S498	5	9	9	3	9	3	9	9	9	9	0.0
D1S2345	8	7	7	6	7	8	7	7	7	7	3.43
D1S305	2	5	8	3	5	2	5	5	5	5	1.73
D1S303	3	3	5	3	3	2	3	3	2/ 3		
	p	m	p	m	p	m	p	m	p	m	
	II:2		II:5; II:6		II:7; II:8		II:9		II:11		

master haplotype   mutation 121-1G>A

mutation 926T>C   mutation 236G>A

Figure 4 Haplotypes of seven of the pycnodysostosis patients (II:2, II:5, II:6, II:7, II:8, II:9 and II:11). The cathepsin K gene (*CTSK*) localised between marker D1S442 and D1S498. 'Master haplotype' and mutations are boxed and marked as shown; p and m indicate the paternal and maternal chromosomes respectively.

Discussion

Pycnodysostosis is a potentially life-threatening genetic disease due to respiratory problems in early infancy. It can be physically invalidating due to multiple complicated bone fractures and may be psychologically devastating due to social isolation and discrimination because of dwarfish height and some very characteristic dysmorphic features, this is in spite of the patients having normal intelligence and often being very well educated.

The advance of knowledge of the pathophysiology of pycnodysostosis will help the patients and their families and may also push forward the work in other related and much more common diseases. The pycnodysostosis candidate gene cathepsin K, CTSK, contributes significantly to the enzymatic activity that has been ascribed to cysteine class proteases, which are implicated in degradation of the protein components within bone during the resorptive stage of organ remodelling.[17] Kinetic and biochemical studies indicate that cathepsin K is intimately involved in the process of bone resorption. Thus it represents a new molecular target in treatment of diseases associated with excessive bone loss – such as osteoporosis.

We have sampled small and primarily unrelated families with pycnodysostosis to look for locus and allele heterogeneity in this disease.

The mutation study reveals two novel mutations 121–1G > A and 926T > C, and one known mutation, 236G > A, in the CTSK gene. Our prediction of the secondary protein structure of 121–1G > A and 926T > C supports an expectation of the two mutations being functional. Also the cathepsin K nucleotide and amino acid sequence is highly conserved among species. The leucine at residue 309 in exon 8 is alike in human, mouse, rabbit, and chicken, and the human amino acid sequence in exon 3 is 97%, 95% and 76% identical to rabbit, mouse and chicken, respectively.[18] This indicates that these mutations are likely to cause disease. The 926T > C mutation is common to all our patients and its frequency in the Danish control population is 0.007 (95% CI: 0.0008–0.0227). Regarding this mutation only, it corresponds to a disease prevalence of 1:22500 (95% CI: from 1: 1.6×10^6 to 1:1940), since there is no tradition of consanguineous marriages in the Danish population. The 121–1G > A and 236G > A mutations respectively are only found in one of the five families. Their frequencies within the population can not be calculated but are predicted to be below 1:300.

The haplotype study finds a rare and highly preserved area, the core haplotype, on chromosome 1q similar for all the patients. A possible common founder in the population studied would explain the very high occurrence of the master haplotype, although in different variations, in the five nuclear families. The means of introduction could either be by a new mutation or by immigration from another population of a person carrying the mutation. The latter cause would predict the mutation to exist in an older form in another population. Thus, we hypothesise that the eight pycnodysostosis patients in our study are related to a common ancestor. Patient II:2 is heterozygous for the core haplotype although homozygous for the 926T > C mutation. This can be explained in two different ways, either the same mutation happened twice in two different chromosomes, or the mutation happened only in one chromosome and the haplotype was changed by crossing over during segregation. The second explanation would suggest the non-core haplotype chromosome in II:2 is much more distantly related than the other seven core-haplotype chromosomes. Our results confirm the disease locus previously reported[6–11] and show that at least three mutations exist in the Danish population. Consequently, this study indicates locus homogeneity and allele heterogeneity in pycnodysostosis within an outbred population. Further more we demonstrate a strong founder effect for pycnodysostosis in Herning County.

Testing more patients with pycnodysostosis will help to estimate a more precise frequency of already found mutations and reveal still unknown ones. Such knowledge will make it possible to test for carrier status in the population and lead to a much more accurate calculation of the mutation frequencies. Attempts to design specific treatment for pycnodysostosis and other diseases with excessive bone loss may benefit from these new results.

References

1 Marotaux P, Lamy M: La pycnodysostosis. *Presse Med* 1962; **70**: 999–1002.
2 Andren L, Dymling JF, Hogeman KE, Wendeberg B: Osteopetrosis-osteolytica: a syndrome of osteopetrosis, acro-osteolysis and open sutures of the skull. *Acta Chir Scand* 1962; **124**: 496–507.
3 Polymeropoulos MH, De Luna RIO, Ide SE, Torres R, Rubenstein J, Francomano CA: The gene for pycnodysostosis maps to human chromosome 1cen-q21. *Nat Gen* 1995; **10**: 238–239.
4 Gelb BD, Edelson JG, Desnick RJ: Linkage of pycnodysostosis to chromosome 1q21 by homozygosity mapping. *Nat Gen* 1995; **10**: 235–237.
5 Gelb BD, Spencer E, Obad S *et al*: Pycnodysostosis: refined linkage and radiation hybrid analyses reduce the critical region to 2 cM at 1q21 and map two candidate genes. *Hum Genet* 1996; **98**: 141–144.
6 Gelb BD, Shi G, Chapman HA, Desnick RJ: Pycnodysostosis, a lysosomal disease caused by cathepsin K deficiency. *Science* 1996; **273**: 1236–1238.
7 Johnson MR, Polymeropoulos MH, Vos HL, De Luna RIO, Francomano CA: A nonsense mutation in the cathepsin K gene observed in a family with pycnodysostosis. *Genome Res* 1996; **6**: 1050–1055.
8 Gelb BD, Willner JP, Dunn TM *et al*: Paternal uniparental disomy for chromosome 1 revealed by molecular analysis of a patient with pycnodysostosis. *Am J Hum Genet* 1998; **62**: 848–854.
9 Punturieri NHA, Francomano C, Weiss S: Compound heterozygosity of CTSK mutations results in pycnodysostosis via an absence of cathepsin K. *Am J Hum Genet* 1998; A365, No. 2113.
10 Hou WS, Bromme D, Zhao Y *et al*: Characterisation of novel cathepsin K mutations in the pro and mature polypeptide regions causing pycnodysostosis. *J Clin Invest* 1999; **103**(5): 731–738.
11 Ho N, Punturieri A, Wilkin D *et al*: Mutations of CTSK result in pycnodysostosis via a reduction in cathepsin K protein. *J Bone Miner Res* 1999; **14**(10): 1649–1653.
12 Miller SA, Dykes DD, Polesky HF: A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 1988; **16**(3): 1215.
13 Nielsen EL: Pyknodysostosis. Review and report of six cases. *Ugeskr Læg* 1973; **135**: 2093–2098.
14 Gelb BD, Shi G, Heller M *et al*: Structure and chromosomal assignment of the human cathepsin K gene. *Genomics* 1997; **41**: 258–262.
15 Geourjon C, Deleage G: SOPM: a self optimised prediction method for protein secondary structure prediction. *Protein Engineering* 1994; **7**: 157–164.
16 Geourjon C, Deleage G: SOPMA: Significant improvements in protein secondary structure prediction by prediction from multiple alignments. *Comput Applic Biosci* 1995; **11**: 681–684.
17 Bossard MJ, Tomaszek TA, Thompson SK *et al*: Proteolytic activity of human osteoclast cathepsin K. *J Biol Chem* 1996; **271**: 12517–12524.
18 Gelb BD, Moissoglu K, Zhang J *et al*: Isolation and characterisation of the murine cDNA and genomic sequence, the homologue of the human pycnodysostosis gene. *Biochem Mol Med* 1996; **59**: 200–206.