Identification of cathepsin C mutations in ethnically diverse Papillon-Lefèvre syndrome patients

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Revised version received
8 September 2000
Accepted for publication
9 September 2000

Abstract

***Introduction*—Papillon-Lefèvre syndrome (PLS) is an autosomal recessive disorder characterised by palmoplantar keratoderma and severe, early onset periodontitis, which results from deficiency of cathepsin C activity secondary to mutations in the cathepsin C gene. To date, 13 different cathepsin C mutations have been reported in PLS patients, all of which are homozygous for a given mutation, reflecting consanguinity.**

***Aim*—To evaluate the generality of cathepsin C mutations in PLS, we studied an ethnically diverse group of 20 unrelated families.**

***Methods*—Mutations were identified by direct automated sequencing of genomic DNA amplified for exonic regions and associated splice site junctions of the cathepsin C gene. Long range PCR was performed to determine the genomic structure of the cathepsin C gene.**

***Results*—The cathepsin C gene spans over 46 kb, with six introns ranging in size from 1.6 to 22.4 kb. Eleven novel mutations and four previously reported mutations were identified in affected subjects from 14 families. Missense mutations were most common (9/15), followed by nonsense mutations (3/15), insertions (2/15), and deletions (1/15). Among these 14 probands, two were compound heterozygotes. Affected subjects with transgressions of the dermal lesions onto the knees or elbows or both had mutations in both the pro- and mature regions of the enzyme, although most were in the mature region.**

***Conclusion*—Mutations in the mature region of cathepsin C were more likely to be associated with the transgressions of the dermatological lesions, although the results were not statistically significant. A comprehensive list of all cathepsin C mutations described to date, representing 25 mutations from 32 families with PLS and related conditions, is also presented.**

(*J Med Genet* 2000;**37**:927–932)

Keywords: cathepsin C; genetics; severe early onset periodontitis; hyperkeratosis

Papillon-Lefèvre syndrome is an inherited palmoplantar keratoderma (PPK) with the characteristic clinical features of palmoplantar hyperkeratosis and severe early onset periodontal destruction.[1 2] The palmoplantar keratodermas (PPKs) are a heterogeneous group of keratinisation disorders primarily characterised by erythema and hyperkeratosis of the palms and soles. More than 40 different types of PPK have been described, and based upon the clinical patterning of palmoplantar dermatological lesions these have been classified into three general groups, diffuse, linear, or focal.[3] In many cases, the palmoplantar lesions represent part of a wider ectodermal defect that includes other cutaneous, mucosal, nail, hair, tooth, and neurological abnormalities. The reclassification of the primary PPKs proposed by Stevens *et al*[4] distinguished those conditions in which the cutaneous abnormalities are restricted to the epidermis (PPK types I, II, and III) from those conditions that presented with a wider ectodermal abnormality (PPK type IV). All of those conditions in which cutaneous lesions are restricted to the epidermis (types I, II, and III) are either acquired or are transmitted as autosomal dominant traits. The Papillon-Lefèvre syndrome (PLS) is an uncommon autosomal recessive type IV palmoplantar ectodermal dysplasia. In addition to the cardinal clinical features of PPK and early onset periodontitis, transgression of skin lesions onto the elbows, knees, and other areas has been noted. A number of additional clinical features have been reported to occur with PLS, including hearing loss, calcification of the dura, follicular hyperkeratosis, nail abnormalities, recurrent infections, and mental retardation.[5 6] Whether these additional clinical findings are related to the primary genetic defect responsible for the cardinal clinical features seen in PLS is unknown. Patients with PLS have been shown to have mutations in both alleles of cathepsin C[7 8] and activity of cathepsin C has been shown to be significantly reduced in carriers of the cathepsin C mutation, and to be virtually nonexistent in patients in whom both alleles of the cathepsin C gene are mutated.[8] PLS has been reported to occur in a diverse range of ethnic groups and parental consanguinity has been noted in more than 50% of these cases.[9] Cathepsin C mutations have been identified in PLS affected subjects from 16 families and parental consanguinity has been reported in 14 of these.[7 8 10 11] The remaining two Lebanese families reported by Toomes *et al*[8] are not reported to be consanguineous; however, the findings of shared haplotypes and homozygosity of cathepsin C mutations in PLS affected

Table 1 Phenotype correlations with CTSC mutations reported to date

*Mutation**	*Predicted effect*	*Location*	*Phenotype†*	*No of families*	*Ethnicity*	*Enzymatic activity‡‡*	*Ref*
199-222del	Deletion of aa 67–74	Exon 2	PLS	1	Chinese¶	220 (parent)	This report
445-446insATGT	Frameshift, termination at aa 157	Exon 3	PLS	1	Bengali		This report
458C→T	T153I	Exon 3	PLS	2	Scottish¶ Eqyptian	300 (parent)	This report
IVS3-1G→A	Altered splicing	Intron 3	PLS§	1	Not specified**		8
622-623insC	Frameshift, termination at aa 223	Exon 4	PLS‡	1	Turkish	2.47 (affected)	This report
628C→T	R210X	Exon 4	PLS§	1	Not specified**		8
704G→A	W235X	Exon 5	PLS	1	Iranian		This report
745G→T	V249F	Exon 5	PLS§	1	Not specified**	18.11 (affected) 347.16 (parent)	8
748C→T	R250X	Exon 5	PLS‡	1	Turkish		This report
755A→T	Q252L	Exon 5	PLS§	1	Not specified**		8
815G→C	R272P	Exon 6	PLS‡	2	Turkish	3.67 (affected)	This report
			PLS§		Not specified**		8
856C→T	Q286X	Exon 6	PLS‡	2	Turkish	4.31 (affected)	7
					Turkish		10
857A→G	Q286R	Exon 6	HMS	1	Indian		10
898G→A	G300S	Exon 7	PLS	1	Vietnamese††		This report
902G→T	G301V	Exon 7	PLS‡	1	Iranian		This report
901G→A	G301S	Exon 7	PLS	2	Iranian		This report
			PLS§		Not specified**		8
910T→A	Y304N	Exon 7	PLS, RP	1	Panamanian		This report
956A→G	E319G	Exon 7	PLS	1	Iranian		This report
1015C→T	R339C	Exon 7	PLS	3	Egyptian		This report
			PLS§		Not specified**		8
			PLS‡		Turkish		11
1019A→G	Y340C	Exon 7	PLS‡	1	Turkish		11
1028-1029delCT	Introduction of premature termination codon	Exon 7	PLS‡	1	Turkish	3.50 (affected)	7
1040A→G	Y347C	Exon 7	PPP	2	Jordanian		11
			PLS§		Not specified**	7.66 (affected) 216.58 (parent)	8
1047delA	Frameshift, termination at aa 349	Exon 7	PLS‡	1	Turkish	Undetectable (affected)	7
1286G→A	W429X	Exon 7	PLS‡	3	Turkish	7.32 (affected)	This report
					Turkish	2.98 (affected)	7
1360A→G	E447G	Exon 7	PLS	1	Vietnamese††		This report

*cDNA numbering considering the initiator Met codon as nucleotide +1. Novel mutations identified in this study are in bold.
†Phenotype symbols: PLS, Papillon-Lefevre syndrome without trangression; PLS‡, Papillon-Lefevre syndrome with trangressions of the hyperkeratotic lesions on elbows/knees; PLS§, Papillon-Lefevre syndrome with presence or absence of trangression unknown; PPP, prepubertal periodontitis; HMS, Haim-Munk syndrome; RP, retinitis pigmentosa.
¶Proband was a compound heterozygote for the 199-222del and 458C→T mutations.
**Families consisted of 3 Egyptian, 3 Indian/Pakistani, and 2 Lebanese.
††Proband was a compound heterozygote for the 898G→A and 1360A→G mutations.
‡‡Activity is expressed as μmol/min/mg protein.

family members suggest that affected members of these families have inherited cathepsin C mutations identical by descent from a common ancestor. Cathepsin C mutations have also been reported in an extended kindred with Haim-Munk syndrome and in a kindred segregating for prepubertal periodontitis without palmoplantar keratosis.[10 11] Parental consanguinity was noted in both these families. The high rate of parental consanguinity observed in PLS cases raises the possibility that the additional clinical findings reported in some cases of PLS may result from homozygosity at other genetic loci and are not, in fact, related to cathepsin C mutations.

To date, 14 different mutations in the cathepsin C gene have been reported for PLS and related conditions.[7 8 10 11] To evaluate the generality of these cathepsin C mutations in PLS, and to evaluate possible genotype-phenotype correlations, we studied the cathepsin C gene in ethnically diverse PLS affected subjects from 20 families. Ethnic backgrounds of those studied included Bengali, Chinese, Egyptian, Iranian, Panamanian, northern European, Turkish, and Vietnamese (table 1).

Toomes *et al*[8] showed that the human cathepsin gene is composed of seven exons, with the exon-intron boundaries exactly matching those of the mouse. They reported exon-intron boundaries, but did not describe the sizes of the specific introns. To clarify the overall genomic structure, we used long range PCR to determine the size of human cathepsin C introns.

Here we describe results of our mutational analysis, including the identification of 11 novel mutations, and also present a summary of cathepsin C mutations identified to date in PLS and related conditions.

Patients and methods

PATIENTS

Twenty four PLS affected subjects, 31 parents, and 14 unaffected sibs from 20 families were ascertained from dental, dermatological, and genetic clinics in Australia, England, Iran, Turkey, and the USA. All subjects provided consent for the study and were clinically examined to determine the presence and location of dermatological lesions, oral findings, and other medical findings.

DNA EXTRACTION

Peripheral venous blood was obtained by standard venepuncture. Genomic DNA was isolated using the QIAamp blood kit (Qiagen Inc).

PCR AMPLIFICATION AND SEQUENCING

Exon 1 was amplified using primer 7-1F[8] as the forward primer and 5'-CTTACCCATAACCG AGCAGTTGAC-3' as the reverse primer. Exons 2-6 were amplified using the primers

Table 2 Primers used to determine genomic organisation of cathepsin C

Region	Primer sequences
Intron 1	F: 5'- TGTCAACTGCTCGGTTATGGGTAA-3'
	R: 5'- TCGAGCTTCTCTTCGTACACCACT-3'
Intron 2	F: 5'- TGACTACAAGTGGTTTGCCTTTTT-3'
	R: 5' TGCTGCCCTCTTCTTTATACTGC-3'
Intron 3	F: 5'- GCCTCTGAGAATGTGTATGTCAAC-3'
	R: 5' CCTGCCCCAAAAATGAGATA-3'
Intron 4	F: 5' TCGAAAAATCCCAAGGTAATC-3'
	R: 5' GGGCCTAGAAAGGAAATATACATT-3'
Intron 5	F: 5' AATTTGTTCGGAACTATTTATTGA-3'
	R: 5' TCGCTTCTAGCATACCCATA-3'

described by Toomes *et al*.[8] Exon 7 was amplified as described before (previously designated exon 2).[7] In our initial paper, the primers listed for exon 1 were mislabelled.[7] The listed forward primer was designed to be used with a reverse primer (5'-GGCTTAGGATTGGGG TCTGA-3') on cDNA templates. The listed reverse primer was designed to be used with a forward primer (5'-GTATGCTAGAAGCGA GAATCCGTAT-3') on genomic DNA templates. Annealing temperatures were 55°C for exons 2 and 5, 58°C for exon 3, 60°C for exons 1 and 4, and 63°C for exons 6 and 7. PCR products were sequenced as described previously.[7]

The genomic organisation of the cathepsin C gene was determined using the primers listed in table 2 and the Expand Long Template PCR System (Boehringer Mannheim). Buffer 3 was used with annealing and extension temperatures of 56°C and 68°C, respectively, and an extension time of 20 minutes. PCR products were separated on a 0.8% agarose gel.

CATHEPSIN C ACTIVITY

Viable leucocyte pellets were obtained from lithium heparinised whole blood by mixing blood with three volumes of 3% dextran (Spectrum Chemical Manufacturing Corp) in normal saline and allowing the red cells to settle for 45 minutes at room temperature. Cells were pelleted by centrifugation at 1500 rpm for five minutes at 4°C. After removal of the supernatant, contaminating red cells were lysed by adding 5 ml cold dH_2O to the pellet for exactly 45 seconds. Five ml of 1.8% saline was then added and the mixture centrifuged at 2000 rpm for five minutes at 4°C. The white cell pellet was washed with 5 ml cold 0.9% saline and centrifuged at 2000 rpm for five minutes at 4°C. Leucocyte pellets were resuspended in dH_2O and sonicated on ice for five seconds each for total of six blasts using a Sonic 300 Dismembrator. Protein concentration was determined by the method of Lowry *et al*.[12]

Enzymatic activity was determined by measuring hydrolysis of the synthetic substrate glycyl-L-arginine-7-amino-4-methylcoumarin (Bachem) at a final concentration of 5 mmol/l using a modified method.[8] All reactions were performed in duplicate. Twenty µl of leucocyte lysate were added to 200 µl of Na_3PO_4 buffer (0.1 mol/l, pH 6.5) in a 96 well plate and then substrate was added. Reactions were allowed to proceed for one hour at room temperature at which time 10 µl of glycine-NaOH buffer (0.5 mol/l, pH 9.8) was added to stop the reaction. Fluorescence was determined using a Perkin-Elmer LS50B luminescence spectrometer at 370 nm excitation and 460 nm emission. The amount of NHMec released was determined by generating a standard curve using NHMec. Cathepsin C activity was reported as µmol NHMec released per minute per mg protein.

GENOTYPE–PHENOTYPE ANALYSIS

The 22 probands on whom genotype and phenotype data were available were subdivided into two categories for both genotype and phenotype. The categories for genotype were mutations in the pro-region and mutations in the mature enzyme. The categories for phenotype were the presence or absence of transgressions of the hyperkeratotic lesions on the knees and elbows. Fisher's exact test was then performed on the resulting 2×2 table. A p value of 0.05 was considered significant.

Results

Clinically, all PLS affected subjects presented with PPK and severe early onset periodontitis. In addition to palmoplantar lesions, dermatological lesions were reported to extend onto the knees and elbows in almost 60% of these patients (table 1). Brittle fingernails were reported in approximately 20% of affected subjects, but this finding was also reported to occur in several unaffected family members and did not appear to segregate with the PLS phenotype. One subject was reported to have ocular findings reminiscent of retinitis pigmentosa in addition to the classical PLS findings. Cytogenetic studies (FISH) of white blood cells from this patient did not suggest a chromosomal deletion that would be consistent with a contiguous gene deletion (data not shown). Seventeen of the 20 families were reported to be consanguineous. The parents of the patient with PLS and retinitis pigmentosa-like findings were unavailable, but the identification of a homozygous cathepsin C mutation, and homozygosity for STRP markers flanking the cathepsin C locus are consistent with consanguinity. The parents of the two other probands were reported to be unrelated.

Of the 20 families studied, mutations were identified in 14, reflecting a 70% detection rate. As shown in table 1, a total of 15 mutations were identified in these families, 11 of which were novel (fig 1). Missense mutations were most common, accounting for 9/15 mutations detected, followed by nonsense mutations (3/15), insertions (2/15), and deletions (1/15).

All families except three were known to be consanguineous before this study. As expected, all 11 probands of consanguineous parents in whom mutations were found were homozygous for their respective mutation. The patient with PLS and retinal changes was found to be homozygous for a 910T→A mutation, consistent with consanguinity. The two non-consanguineous probands, both Australian, were compound heterozygotes, one for the 199-222del and 458C→T mutations (family 1) and the other for the 898G→A and 1360A→G mutations (family 2). These subjects represent

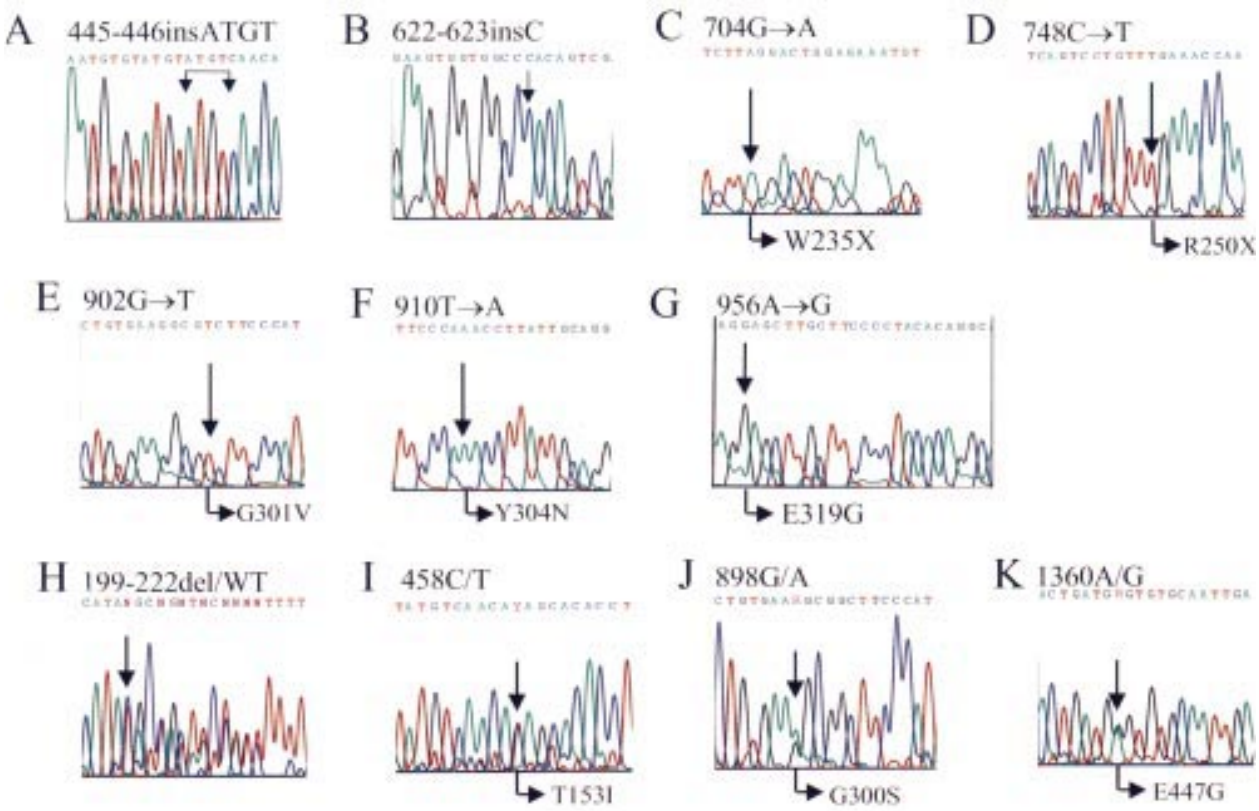

Figure 1 Eleven novel mutations in CTSC. Mutations A-G were present in the homozygous state in affected subjects. Mutations H and I (family 1) and J and K (family 2) were present in the compound heterozygous state. (A) 445-446insATGT, which results in a frameshift and the introduction of a stop codon at position 157. (B) 622-623insC, which results in a frameshift with a stop codon introduced at amino acid position 223. (C) 704G→A, which results in W235X. (D) 748C→T, which results in R250X. (E) 902G→C, which results in G301V. (F) 910T→A, which results in Y304N. (G) 956A→G, which results in E319G. (H) 199-222del, which results in deletion of amino acids 67-74. (I) 458C→T, which results in T153I. (J) 898G→A, which results in G300S. (K) 1360A→G, which results in E447G.

the first identification of compound heterozygosity in PLS. Enzyme analysis of the parents from family 1 indicated that each had approximately 50% of normal cathepsin C activity, consistent with their carrier status. The father and mother had specific activities of 220 and 300 μmol/min/mg, respectively (control range 685-1200 μmol/min/mg). Sequence analysis showed that the father was the carrier of the 199-222del mutation, while the mother was the carrier of the 458C→T mutation. Enzyme analysis was also conducted on seven probands, all of whom had specific activities of less than 5% of the control range (table 1).

Within this study population, only one mutation was found in more than one family. The 458C→T mutation was found in two families, one a consanguineous Egyptian family and the other an Australian family of Scottish ancestry.

All probands with transgression of the dermatological lesions onto the elbows and knees had mutations in exons 4-7, corresponding to the 10 kDa pro-region and the heavy and light chains of the mature region. There was no correlation between the type of mutation (missense, nonsense, insertion, etc) and the presence or absence of the lesions on the knees or elbows or both. The patients with mutations in exons 2 and 3 have typical PLS and do not have transgression of the lesions. Fisher's exact test was used to conduct a formal genotype-phenotype analysis. A 2 × 2 table was constructed for 22 probands. Genotypes were classified as mutations occurring in the pro-region of cathepsin C or in the mature enzyme, and phenotypes were classified as the presence or absence of transgressions of the hyperkeratotic lesions on the elbows or knees or both. A total of 13 patients had trangressions, with 12 of the 13 having mutations in the mature region. Of the nine patients without trangressions, five of the nine had mutations in the mature region. The p value by Fisher's exact test was 0.12.

Results of long range PCR performed to determine the genomic organisation of the human cathepsin C gene showed that it spans approximately 46 kb, with six introns varying in size from 1.6 to 22.4 kb (fig 2). Our experimental results are consistent with subsequently released sequence data for BAC 292e14 (Accession number AC011088).

Discussion

Cathepsin C, or dipeptidyl aminopeptidase I (E.C. 3.4.14.1), is a lysosomal cysteine protease that removes dipeptides from the amino terminus of protein substrates. At higher pH, it also exhibits dipeptidyl transferase activity.[13] Cathepsin C differs from other cathepsins in that it is an oligomeric enzyme composed of four identical subunits.[14 15] As shown in fig 2, each subunit contains three different polypeptide chains, the heavy chain, light chain, and the pro-region. These chains are held together

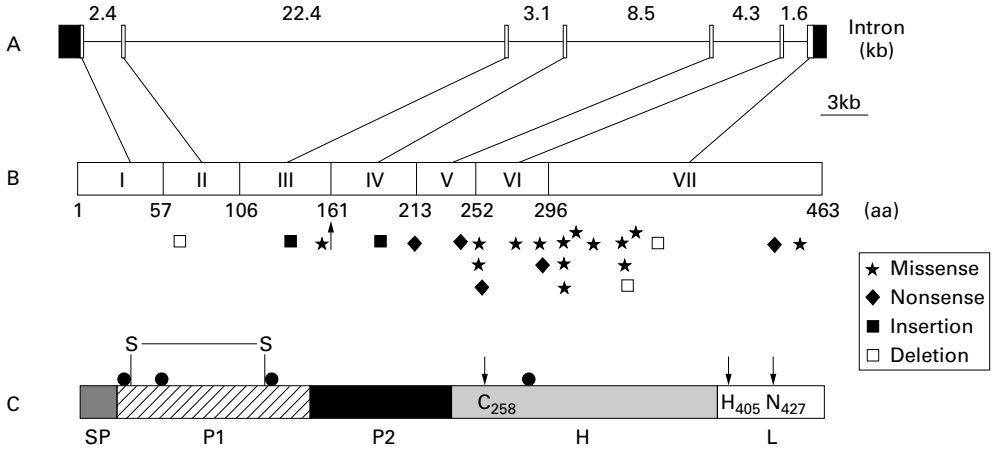

Figure 2 Mutations of the CTSC gene. (A) Genomic structure of the CTSC gene with introns shown as solid lines and exons depicted as boxes. The 5' and 3' untranslated regions are shown as filled boxes. (B) Coding region of the CTSC gene. The amino acid numbers are shown at the end of each exon. Mutations listed in table 1 are shown according to their genomic locations. The splicing site mutation is indicated by an arrow. (C) Subunit structure of CTSC polypeptide with SP, signal peptide; P1, 13.5 kDa pro-region; P2, 10 kDa, pro-region; H, heavy chain; L, light chain. The 10 kDa pro-region is cleaved out upon activation. The disulphide bond within the 13.5 pro-region is shown. The glycosylation sites are indicated by filled circles. Arrows indicate the active sites.

by non-covalent interactions.[16] The C-terminal portion of the pro-region is cleaved out from the pro-region on activation. The residual pro-region is heterogeneous with a proportion being intact and the remainder being cleaved at position 58 or 61, resulting in two small peptides held together by a disulphide bond. It has been postulated that the pro-region is important in the structure and activity of the mature enzyme. The oligomerisation and protein splicing appears to occur in the endoplasmic reticulum-Golgi intermediate compartment.[17] All four potential N-glycosylation sites are glycosylated.[16]

The cathepsin C gene was originally reported to consist of two exons spanning 3.5 kb.[18] Toomes *et al*[8] showed that the human cathepsin C gene is composed of seven exons, with the exon-intron boundaries exactly matching those of the mouse. This finding explains the difficulty that we had amplifying "exon 1" except from cDNA. In this report, we show that the cathepsin C gene actually spans over 46 kb, with the smallest intron being 1.6 kb and the largest 22.4 kb (fig 2).

Mutations in the cathepsin C gene have been shown to result in Papillon-Lefèvre syndrome (PLS), Haim-Munk syndrome (HMS), and prepubertal periodontitis (PPP). The common finding in all three conditions is severe early onset periodontitis. The finding that the same mutation, 1040A→G, results in two distinct phenotypes, PLS or PPP, indicates that other factors, genetic or environmental, play a role in the ultimate phenotype. Clinical findings reported for families with HMS vary from classical findings in PLS to PPK, severe early onset periodontitis, onychogryposis, pes planus, arachnodactyly, and acro-osteolysis. While the same cathepsin C mutation (exon 6, 2127A→G) is reported in all HMS patients, the clinical phenotype is reported to vary in different nuclear families from the same kindred.[19 20] These findings suggest that other genetic/environmental factors may be important determinants of the clinical phenotype observed. To date, a total of 25 mutations have been described in the cathepsin C gene, with single base changes accounting for 80% (20/25) of mutations. All of the missense mutations, except for T153I, occur at highly conserved residues. The T153I mutation occurs at a residue conserved between humans and dogs and is in the portion of the pro-region that is normally cleaved out upon activation.[17 21] Given that the T153I mutation occurs within 10 amino acids of the N-terminal cleavage site, it is attractive to hypothesise that the mutation interferes with or prevents this normal processing. Seven mutations have been found in more than one family (table 1). Mutations 1015C→T and 1286G→A have each been observed in three families. Although the 856C→T mutation has been described in two Turkish kindreds, affected members of these families do not share a common haplotype for genetic loci flanking the *CTSC* gene, suggesting that the mutations have arisen independently.

The only distinguishing feature between the subjects with PLS was the presence or absence of dermatological lesions on the knees/elbows. There was no correlation between the type of mutation (missense, insertion, etc) and the presence or absence of trangression of the lesions. Although 77% (17/22) of the mutations identified in our laboratory occurred in the mature region, 92% (12/13) of the mutations associated with transgression occurred in this region compared to 56% (5/9) of the mutations not associated with transgressions. Analysis of the location of mutation (pro-region versus mature region) by Fisher's exact test suggests that mutations in the mature region are more likely to be associated with transgression of the lesions, although the results were not statistically significant ($p=0.12$). As the underlying mutation is discovered in additional PLS patients, further evaluation of the genotype-phenotype correlation will be possible.

One patient had PLS and eye findings suggestive of retinitis pigmentosa. No other

PLS patients were reported to have eye changes. That the subject with ocular findings is homozygous for the 910T→A mutation supports the notion of consanguinity in this child. The probability of manifesting a recessive disorder is increased in consanguineous matings owing to the sharing of alleles identical by descent. Given these findings, we hypothesise that the retinitis pigmentosa in this proband is probably autosomal recessive in nature and is most likely unrelated to the cathepsin C mutation.

Of the 36 families with PLS that have been studied to date, mutations have been found in 30 families, providing a detection rate of approximately 83%. The six families in whom we did not find a mutation are consanguineous, making it unlikely that a heterozygous change was missed. Additionally, four of these families were part of a linkage study that localised the PLS gene to chromosome 11q14, within a candidate interval of less than 5 cM.[22] None of these families provided any evidence for genetic heterogeneity, and all were consistent with linkage to this genetic interval on chromosome 11q. Although the possibility of genetic heterogeneity cannot be excluded, it is possible that intronic or regulatory mutations of the cathepsin C gene may account for the phenotype in these six families. Of the 25 cathepsin C mutations identified, most cluster in the portion of the gene coding for the heavy chain region of cathepsin C (exons 5, 6, and 7). However, the presence of mutations in exons 2, 3, and 4 suggest that mutational analyses of the cathepsin C gene will require evaluation of the whole gene. Future studies will also indicate whether or not mutations in the mature region of the enzyme are more likely to be associated with trangressions of the dermatological lesions onto the elbows/knees.

These studies were supported in part by National Institutes of Dental and Craniofacial Research R01-DE12920. The authors are grateful to Dr Eleanor Feingold for assistance with statistical analysis.

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