Substitution of cysteine for glycine at residue 415 of one allele of the $\alpha 1(I)$ chain of type I procollagen in type III/IV osteogenesis imperfecta

A C Nicholls, J Oliver, D V Renouf, M Keston, F M Pope

Abstract

We have examined the type I collagen in a patient with type III/IV osteogenesis imperfecta. Two forms of $\alpha 1(I)$ chain were produced, one normal and the other containing a cysteine residue within the triple helical domain of the molecule. Cysteine is not normally present in this domain of type I collagen. Peptide mapping experiments localised the mutation to peptide $\alpha 1(I)CB3$ which spans residues 403 to 551 of the triple helix. Subsequent PCR amplification of cDNA covering this region followed by sequencing showed a G to T single base change in the GGC codon for glycine 415 generating TGC, the codon for cysteine. The effect of the mutation on the protein is to delay secretion from the cell, reduce the thermal stability of the molecule by 2°C, and cause excessive post-translational modification of all chains in molecules containing one or more mutant $\alpha 1(I)$ chains. The clinical phenotype observed in this patient and the position of the mutation conform to the recent prediction of Starman *et al* that Gly→Cys mutations in the $\alpha 1(I)$ chain have a gradient of severity decreasing from the C-terminus to the N-terminus.

Osteogenesis imperfecta (OI) is an inherited connective tissue disease typified by excessive bone fragility. The major structural component of bone is

type I collagen and molecular abnormalities of this protein have been identified in a number of OI patients. These abnormalities include major gene deletions,^{1,2} gene insertions,³ and mutations causing exon skipping,^{4,5} but most have been point mutations of glycine codons in either the $\alpha 1(I)$ or $\alpha 2(I)$ genes that disrupt the normal Gly-X-Y repeat sequence and destabilise the type I collagen molecule to varying degrees⁶⁻¹⁹ or occur in non-helical domains.²⁰⁻²²

Osteogenesis imperfecta is a heterogeneous syndrome which has to date been subclassified by clinical and radiological features as suggested by Silience *et al*.²³ Only now, as more mutations are being identified, is it becoming feasible to correlate disease phenotype with the molecular abnormalities and their effect on the properties of the collagen molecule.

We report here the clinical phenotype and characterisation of the molecular defect of a patient with moderately severe osteogenesis imperfecta (OI III/IV) in whom one allele for the pro $\alpha 1(I)$ chain of type I collagen harbours a glycine→cysteine mutation at position 415. (Numbering of residues follows the normal convention of beginning with the first residue of the triple helix.)

Materials and methods

CLINICAL SUMMARY

The patient was a male in his late fifties (fig 1). He was the only affected child of normal parents, with 10 sibs both younger and older than himself. He had a normal birth weight and length. His first recorded fracture occurred at 6 weeks of age. Over the next 16 years he suffered more than 270 fractures leading to progressive deformity of his skeleton. His sclerae were reported 'bluish' at birth but have become paler with age. (This is characteristic of type III OI.) He developed a conductive hearing loss in the third decade of life (not described previously in type III or type IV OI). Now edentulous, his teeth were reportedly yellowish-brown, suggesting dentinogenesis imperfecta. The skeletal radiographs (fig 2) showed generalised osteopenia with multiple fractures throughout the skeleton, including wedge

Dermatology Research Group, Clinical Research Centre, Watford Road, Harrow, Middlesex HA1 3UJ.
A C Nicholls, J Oliver, D V Renouf*, F M Pope

MRC Clinical and Population Cytogenetics Unit, Edinburgh.
M Keston

*Present address: Glycoconjugates Research Section, Clinical Research Centre, Harrow.

Correspondence to Dr Nicholls.

Received for publication 11 April 1991.
Revised version accepted for publication 7 May 1991.

Figure 1 Clinical appearance of the patient in this study.

compression fractures of the vertebral bodies. There was bowing and deformity of the long bones secondary to bone softening and a marked platybasia was noted.

CELL CULTURE STUDIES

Skin fibroblast cultures were established and maintained in Dulbecco's MEM medium (Flow Laboratories, Irvine, Scotland). Labelling of the cells with ^{14}C -proline was normally carried out at $1\ \mu\text{Ci ml}^{-1}$ as previously described.²⁴ In experiments with short labelling times, the isotope concentration was increased to $5\ \mu\text{Ci ml}^{-1}$ and for pulse chase experiments the initial pulse contained $2\ \mu\text{Ci ml}^{-1}$ isotope. Before labelling with ^{35}S -cystine, confluent cells were briefly incubated with cystine free Dulbecco's MEM and then labelled for 24 hours with the same medium containing $10\ \mu\text{Ci ml}^{-1}$ ^{35}S -cystine. Cells were incubated in a humidified atmosphere of 5% CO_2 , 95% air at 37°C .

After labelling, medium and cells were harvested separately. The medium was mixed with proteinase inhibitors, N-ethylmaleimide ($50\ \mu\text{g ml}^{-1}$), phenylmethyl sulphonyl fluoride ($50\ \mu\text{g ml}^{-1}$), and EDTA ($0.01\ \text{mol/l}$). Washed cells were trypsinised from the plate, pelleted, and lysed in either phosphate buffered saline containing 0.5% triton X100 (PBS-triton) or 0.5 mol/l acetic acid containing 0.5% triton X100 (acid-triton).

Proteins were precipitated from medium or PBS-triton cell extracts by addition of two volumes of

absolute ethanol and incubation at 4°C overnight. Precipitates were collected by centrifugation at 6500 rpm for five minutes at 4°C in a microfuge. Pellets were washed three times with 66% aqueous ethanol, drained, and dried before redissolving in 0.5 mol/l acetic acid or SDS gel electrophoresis sample buffer.

Pepsin digestion of alcohol precipitates or acid-triton cell extracts was carried out in 0.5 mol/l acetic acid with $50\ \mu\text{g ml}^{-1}$ enzyme for six hours at 15°C . Digestion was stopped by addition of $0.5\ \mu\text{g ml}^{-1}$ pepstatin and lyophilisation.

Thermal stability measurements were carried out on ^{14}C -proline labelled samples essentially as described.^{25,26}

Cyanogen bromide peptide mapping of α chains separated by gel electrophoresis was performed by the method of Barsh *et al*²⁶ using whole tracks or individual bands cut from the gel.²⁷

GEL ELECTROPHORESIS

Procollagen and collagen chains were separated on 5% polyacrylamide gels containing 2 mol/l urea using the buffer system of Laemmli.²⁸ Cyanogen bromide peptides were separated on 10% acrylamide gels containing 0.5 mol/l urea. Radioactive peptides were visualised by autoradiography or fluorography²⁹ using Fuji or Kodak x ray film.

ISOLATION OF CYTOPLASMIC RNA

Cytoplasmic RNA was isolated from cultured fibroblasts ($3 \times 150\ \text{mm}$ Petris) fed with medium containing fresh ascorbic acid 24 hours before harvesting. Extraction of RNA used the NP40 lysis technique.^{24,30}

cDNA SYNTHESIS AND PCR AMPLIFICATION

Primers used for PCR amplification were: 5' sense primer 5'-CCTGGTCCTGATGGCAAACT-3' which corresponds to sequences within CB8 of the $\alpha 1(\text{I})$ mRNA and 3' antisense primer 5'-TTTGGATCCGGAAGACCAGCTGCACCAG-3' which is complementary to sequences within CB7 of the $\alpha 1(\text{I})$ mRNA.

First strand cDNA synthesis used $5\ \mu\text{g}$ total RNA in $25\ \mu\text{l}$ of $1 \times \text{RT}$ buffer (50 mmol/l Tris HCl, pH 8.3, at 42°C , 50 mmol/l KCl, 10 mmol/l MgCl_2 , 1 mmol/l DTT, $10\ \mu\text{g ml}^{-1}$ BSA) containing 4 mmol/l sodium pyrophosphate, 20 units RNasin, 1 mmol/l each dNTP, and 100 ng of 3' antisense primer. Incubation with 20 units of AMV reverse transcriptase (Pharmacia) continued for 90 minutes at 42°C . One fifth of this reaction product was amplified for 40 cycles at 94°C for two minutes, 55°C for two minutes, and 65°C for two minutes using a $100\ \mu\text{l}$ reaction containing $1 \times \text{PCR}$ buffer (10 mmol/l Tris

Figure 2 Skeletal radiographs of (A) skull, (B) spine, and (C) tibiae of patient.

HCl, pH8.4, 50 mmol/l KCl, 1.5 mmol/l $MgCl_2$, 400 μ mol/l each dNTP, 100 ng each 5' and 3' primer, and 2.5 U *Taq* polymerase (Cetus). The reaction products were gel purified, blunt end ligated into M13mp18, and sequenced by the dideoxy chain termination method³¹ using sequenase (USB).

Results

Gel electrophoresis of pepsin derived medium collagens of cells from this patient (fig 3) showed an additional high molecular weight component migrating between $\alpha 1(III)_3$ and $\alpha 1(I)$. There was also evidence of slow migration of the $\alpha 1(I)$ band in the patient compared to control collagens. If

samples were reduced with mercaptoethanol before electrophoresis both the abnormal component and the $\alpha 1(III)_3$ band disappeared without the appearance of any new bands (data not shown). Two dimensional electrophoresis of collagens run first unreduced then after reduction with mercaptoethanol (fig 4) showed that both $\alpha 1(III)_3$ and the abnormal component migrated with the mobility of the $\alpha 1(I)$ after reduction. This suggested that the high molecular weight component was a disulphide bonded dimer of α chains.

The nature of these α chains was investigated by in situ peptide mapping with cyanogen bromide (fig 5). The pattern for the dimer (*in fig 5A) clearly resembled that of $\alpha 1(I)$ but there were some significant differences. Instead of a doublet for the $\alpha 1CB7$

Figure 3 Sodium dodecyl sulphate gel electrophoresis of pepsin treated medium collagens from normal fibroblasts (C) and the patient's skin fibroblasts (P). *Denotes abnormal band.

Figure 4 Two dimensional gel electrophoresis of collagens from the patient. Samples were first electrophoresed without reduction (a), the tracks were equilibrated with sample buffer containing mercaptoethanol, and re-electrophoresed in the same concentration gel (b).

and $\alpha 1\text{CB}8$, only a single broad spot was observed in the position of $\alpha 1\text{CB}7$. The $\alpha 1\text{CB}3$ spot was very faint and there was an additional spot above $\alpha 1\text{CB}7$ (arrowed in fig 5A, -MSH) not normally present in $\alpha 1(\text{I})$ maps (cf fig 5A and C, -MSH). Examination of the $\alpha 1(\text{I})$ region from the patient showed tilting of the bands for $\alpha 1\text{CB}8$, and the partial cleavage products $\alpha 1\text{CB}5-8$ and $\alpha 1\text{CB}4-5-8$ (seen very faintly above $\alpha 1\text{CB}7$) suggesting heterogeneity of these components owing to excessive post-translational modification.²² Other peptides appeared normal.

If after cyanogen bromide digestion the sample was reduced with mercaptoethanol before electrophoresis (fig 5B, + MSH) the spot arrowed in fig 5A disappeared and there was a decrease in the amount of partial cleavage products at the top of the gel but, unexpectedly, there was no major alteration to other peptides. These data suggested that the substitution was in $\alpha 1\text{CB}3$, but with $\alpha 1\text{CB}3$ not appearing after reduction the interpretation was not unequivocal. Cells were therefore labelled with ^{35}S -cysteine, the procollagens precipitated, pepsin digested, and electrophoresed with and without previous reduction with mercaptoethanol (fig 6A). Unlike ^{14}C -proline labelling, where collagen is the predominant labelled species because of its high proline content, after labelling with ^{35}S -cysteine the collagens were only a minor component of the total labelled protein even after pepsin digestion. When run unreduced (fig 6A, lane 1) there were several high molecular weight bands, but the $\alpha 1(\text{I})$ dimer (*) and $\alpha 1(\text{III})_3$ were clearly resolved. After reduction (fig 6A, lane 3) the mutant $\alpha 1(\text{I})$ and $\alpha 1(\text{III})_3$ ran together at the position of $\alpha 1(\text{I})$; all other labelled species migrated ahead of them in the gel. Bands corresponding to the ^{35}S labelled $\alpha 1$ dimer (*), $\alpha 1(\text{III})_3$ (lane 1), and the $\alpha 1(\text{I})$ region of the reduced sample (lane 3), as well as ^{14}C -proline labelled $\alpha 1(\text{III})_3$, $\alpha 1(\text{I})$, and $\alpha 2(\text{I})$ (lane 2), were excised and digested in situ with cyanogen bromide. In the maps obtained (fig 6B) the ^{35}S labelled $\alpha 1$ dimer (lane c) showed a single band penetrating the gel (arrowed) migrating with the same relative mobility as the extra spot seen in the ^{14}C -proline labelled maps (fig 5). There was also a significant amount of activity that did not penetrate the gel, which was assumed to be partial cleavage products containing disulphide bridges. In the reduced sample (lane d, fig 6B) several spots were seen and most of them were attributable to the type III collagen which contains two cysteine residues in its C-terminal peptide $\alpha 1(\text{III})\text{CB}9$ and its partial cleavage products, for example, $\alpha 1(\text{III})\text{CB}5-9$ (lanes d and e, fig 6B). In addition, there was a prominent spot in the position of $\alpha 1(\text{I})\text{CB}3$ (large arrow, lane d) and a slightly less prominent spot corresponding to $\alpha 1(\text{I})\text{CB}8-3$ (small arrow, lane d). This unequivocally localised the mutant cysteine to the CB peptide $\alpha 1\text{CB}3$ which

Figure 5 Two dimensional peptide mapping by in situ cyanogen bromide digestion of ^{14}C -proline labelled medium collagens from the patient (A,B) and control (C,D) fibroblasts. Samples were run unreduced in the first dimension, cyanogen bromide digested, then equilibrated with sample buffer without (A,C) or with (B,D) mercaptoethanol before electrophoresis in the second dimension. *marks abnormal component of the patient's collagens. Note spot arrowed in panel (A) disappears upon reduction (open circle in panel B). Note also tilting of $\alpha 1\text{CB}8$ and other bands in the $\alpha 1(I)$ pattern of the patient (A,B) not seen in control $\alpha 1(I)$ (panels C,D).

contains residues 403 to 551 of the $\alpha 1(I)$ chain (fig 6C).

To identify the precise mutation, cDNA for this region was amplified by PCR using flanking primers (fig 6C). A single fragment of 577 bp was obtained and subcloned into M13mp18. Several clones were picked and two distinct sequences were obtained in approximately equal abundance. One was normal but the other had the GGC codon for glycine 415 changed to TGC, the codon for cysteine (fig 7). After sequencing the entire fragment, only one other difference from the published sequence was observed. All clones had the sequence GGT for residue 517 instead of the published GGA. This change did not alter the protein sequence, being the third base of a glycine residue.

The effect of the mutation on certain physical and biological properties of the molecule was also investigated. The thermal stability of the molecule was reduced by approximately 2°C compared to con-

trols (data not shown). Pulsing cells with isotope for periods up to four hours and analysing the medium and cells separately indicated that mutant molecules were poorly secreted and tended to accumulate within the cell (data not shown).

Discussion

Clinically the patient is difficult to classify according to the criteria of Sillence *et al.*²³ He has a progressively deforming OI compatible with either OI type III or OI type IV phenotypes. With 10 normal sibs he is clearly a sporadic case, consistent with heterozygosity for a new dominant collagen mutation. Our analysis of the collagen produced by the patient's dermal fibroblasts identified the substitution of a cysteine residue in the triple helical domain of one $\alpha 1(I)$ allele. The incorporation of two mutant chains in a type I collagen molecule (chain composition $\alpha 1_2\alpha 2$) leads to the formation of disulphide bonded

Figure 6 (A) Gel electrophoresis of ^{35}S -cysteine labelled proteins. The patient's ^{35}S -cysteine labelled proteins were run unreduced (lane 1) or reduced (lane 3). ^{14}C -proline labelled control fibroblast collagens were run unreduced (lane 2). The unreduced mutant is marked *. When reduced it comigrates with $\alpha 1(III)$ to the position of $\alpha 1(I)$. (B) In situ cyanogen bromide digestion of bands cut from the gel in fig 6A. Lanes (a) and (b) are ^{14}C -proline labelled $\alpha 1(I)$ and $\alpha 2(I)$ chains respectively. Lane (c) is the ^{35}S labelled mutant dimer *, lane (d) is the ^{35}S labelled $\alpha 1(I)$ region from lane 3, fig 6A, lane (e) is ^{35}S labelled $\alpha 1(III)$ from lane 1, fig 6A, lane (f) is ^{14}C -proline labelled $\alpha 1(III)$ from lane 2, fig 6A. (C) Linear cyanogen bromide peptide map of the $\alpha 1(I)$ chain showing location of primers used for PCR amplification of patient's mRNA.

$\alpha 1$ dimers. Peptide mapping experiments with ^{14}C -proline labelled proteins, while confirming the mutant was an $\alpha 1(I)$ chain, could not positively identify the site of the substitution. Two peptides $\alpha 1CB8$ and $\alpha 1CB3$ were missing from the dimer but, unexpectedly, neither reappeared on reduction of the disulphide bond. In retrospect this was probably owing to a combination of overmodification of $\alpha 1CB8$, causing it to migrate with $\alpha 1CB7$, and inefficient cleavage at the methionine residue between $\alpha 1CB7$ and $\alpha 1CB3$, possibly as a result of the proximity of the disulphide bond to the methionine (13 residues). However, specific labelling of the mutation with ^{35}S -cysteine showed $\alpha 1CB3$ (residues

403 to 551 of the triple helical domain) contained the substitution. Analysis of the $\alpha 1(I)$ mRNA for this region by PCR amplification of the cDNA showed heterozygosity for a single base change from a G to a T, altering the GGC codon for glycine 415 to TGC, the codon for cysteine. This substitution disrupts the Gly-X-Y-Gly-X-Y repeat sequence essential for the formation of a stable triple helical domain. As a result, type I collagen molecules containing mutant α chains have a reduced thermal stability ($2^\circ C$ below normal), have excessive post-translational modification amino-terminal of the substitution owing to delayed helix formation,²⁵ and are poorly secreted from the cell. All these factors would combine to

produce an inadequate extracellular matrix and since type I collagen is the major structural component of bone would result in the osteopenia and bone fragility observed in this patient.

A number of Gly→Cys substitutions have now been identified in the $\alpha 1$ chain of type I collagen from OI patients, and the severity of the disease seems to depend on the position of the mutation within the molecule. Recently Starman *et al*¹² have contrasted the phenotypic features of three such mutations. A Gly→Cys mutation at residue 718 (and others further C-terminal) resulted in lethal type II OI. Similar substitutions at residue 526 gave the OI III phenotype and at residue 94 produced the OI I phenotype. The results we have obtained with this patient are consistent with these observations and suggest that the varying clinical phenotype reflects subtle changes in the physical and biological properties of the mutant molecules.

(B)

Mutation in cDNA

Normal $\alpha 1CB3$

ATG GGA TTC CCT GGA CCT AAA GGT GCT GCT GGA GAG CCC GGC AAG GCT GGA
Met Gly Phe Pro Gly Pro Lys Gly Ala Ala Gly Glu Pro Gly Lys Ala Gly

Mutant $\alpha 1CB3$

ATG GGA TTC CCT GGA CCT AAA GGT GCT GCT GGA GAG CCC TGC AAG GCT GGA
Met Gly Phe Pro Gly Pro Lys Gly Ala Ala Gly Glu Pro Cys Lys Ala Gly
res 415

We gratefully acknowledge the assistance of Mrs O Cutting in maintaining the cell lines, the Medical Illustration Department, Clinical Research Centre for the figures, and Mrs Teresa Barrett for typing the manuscript.

- 1 Chu ML, Gargiulo V, Williams CJ, Ramirez F. Multiexon deletion in an osteogenesis imperfecta variant with increased type III collagen mRNA. *J Biol Chem* 1985;260:691-4.
- 2 Barsh GS, Roush CL, Bonadio J, Byers PH, Gelinas RE. Intron mediated recombination may cause a deletion in an $\alpha 1$ type I collagen chain in a lethal form of osteogenesis imperfecta. *Proc Natl Acad Sci USA* 1985;82:2870-4.
- 3 Byers PH, Starman BJ, Cohn DH, Horwitz AL. A novel mutation causes a perinatal lethal form of osteogenesis imperfecta—an insertion in one $\alpha 1(I)$ allele (COL1A1). *J Biol Chem* 1988;263:7855-61.
- 4 Tromp G, Prockop DJ. Single base mutation in the pro $\alpha 2(I)$ collagen gene that causes efficient splicing of RNA from exon 27 to exon 29 and synthesis of a shortened but inframe pro $\alpha 2(I)$ chain. *Proc Natl Acad Sci USA* 1988;85:5254-8.
- 5 Kuivaniemi H, Sabol C, Tromp G, Sippola-Thiele M, Prockop DJ. A 19-base pair deletion in the pro $\alpha 2(I)$ gene of type I procollagen that causes in-frame RNA splicing from exon 10 to exon 12 in a proband with atypical osteogenesis imperfecta and his asymptomatic mother. *J Biol Chem* 1988;263:11407-13.
- 6 Cohn DH, Byers PH, Steinmann B, Gelinas RE. Lethal osteogenesis imperfecta resulting from a single nucleotide change in one human pro $\alpha 1(I)$ collagen allele. *Proc Natl Acad Sci USA* 1986;83:6045-7.
- 7 de Vries WN, de Wet W. The molecular defect in an autosomal dominant form of osteogenesis imperfecta. Synthesis of type I procollagen containing cysteine in the triple helical domain of pro $\alpha 1(I)$ chains. *J Biol Chem* 1986;261:9056-64.
- 8 Bateman JF, Chan D, Walker ID, Rogers JG, Cole WG. Lethal perinatal osteogenesis imperfecta due to the substitution of arginine for glycine at residue 391 of the $\alpha 1(I)$ chain of type I collagen. *J Biol Chem* 1987;262:7021-7.
- 9 Bateman JF, Lamande S, Dahl HHM, Chan D, Cole WG. Substitution of arginine for glycine 664 in the collagen $\alpha 1(I)$ chain in lethal perinatal osteogenesis imperfecta. *J Biol Chem* 1988;263:11627-30.
- 10 Vogel BE, Doelz R, Kadler KE, Hojima Y, Engel J, Prockop DJ. A substitution of cystine for glycine 748 of the

Figure 7 (A) DNA sequencing gel of cDNAs obtained by PCR amplification of $\alpha 1CB3$ of the patient's $\alpha 1(I)$ mRNA. (B) cDNA and amino acid sequence obtained for normal and mutant alleles.

- $\alpha 1(I)$ chain produces a kink at this site in the procollagen I molecule and an altered N-proteinase cleavage site over 225nm away. *J Biol Chem* 1988;263:19248-55.
- 11 Wenstrup RJ, Cohn DH, Cohen T, Byers PH. Arginine for glycine substitution in the triple helical domain of the products of one $\alpha 2(I)$ collagen allele (COL1A2) produces the osteogenesis imperfecta type IV phenotype. *J Biol Chem* 1988;263:7734-40.
 - 12 Starman BJ, Eyre D, Charbonneau H, et al. Osteogenesis imperfecta. The position of substitution for glycine by cysteine in the triple helical domain of the pro $\alpha 1(I)$ chains of type I collagen determines the clinical phenotype. *J Clin Invest* 1989;84:1206-14.
 - 13 Patterson E, Smiley E, Bonadio J. RNA sequence analysis of a perinatal lethal osteogenesis imperfecta mutation. *J Biol Chem* 1989;264:10083-7.
 - 14 Marini JC, Grange DK, Gottesman GS, Lewis MB, Koeplin DA. Osteogenesis imperfecta type IV. Detection of a point mutation in one $\alpha 1(I)$ collagen allele (COL1A1) by RNA/RNA hybrid analysis. *J Biol Chem* 1989;264:11893-900.
 - 15 Pack M, Constantinou CD, Kalia K, Nielsen KB, Prockop DJ. Substitution of serine for $\alpha 1(I)$ glycine 844 in a severe variant of osteogenesis imperfecta minimally destabilizes the triple helix of type I procollagen. *J Biol Chem* 1989;264:19694-9.
 - 16 Constantinou CD, Nielsen KB, Prockop DJ. A lethal variant of osteogenesis imperfecta has a single base mutation that substitutes cysteine for glycine 904 of the $\alpha 1(I)$ chain of type I procollagen. *J Clin Invest* 1989;83:574-84.
 - 17 Lamande SR, Dahl HMM, Cole WG, Bateman JF. Characterization of point mutations in the collagen COL1A1 and COL1A2 genes causing lethal perinatal osteogenesis imperfecta. *J Biol Chem* 1989;264:15809-12.
 - 18 Baldwin CT, Constantinou CD, Damars KW, Prockop DJ. A single base mutation that converts gly 907 in the $\alpha 2(I)$ chain of type I procollagen to aspartate in a lethal variant of osteogenesis imperfecta. *J Biol Chem* 1989;264:3002-6.
 - 19 Prockop DJ, Constantinou CD, Dombrowski KE et al. Type I procollagen: the gene-protein system that harbours most of the mutations causing osteogenesis imperfecta and probably more common heritable disorders of connective tissue. *Am J Med Genet* 1989;34:60-7.
 - 20 Nicholls AC, Pope FM, Craig D. An abnormal collagen α chain containing cysteine in autosomal dominant osteogenesis imperfecta. *BMJ* 1984;24:112-3.
 - 21 Cohn DH, Apone S, Eyre DR et al. Substitution of cysteine for glycine within the carboxyl-terminal telopeptide of the $\alpha 1(I)$ chain of type I collagen produces osteogenesis imperfecta. *J Biol Chem* 1988;263:14605-7.
 - 22 Labherd ME, Wirtz MK, Pope FM, Nicholls AC, Hollister DW. A cysteine for glycine substitution at position 1017 in an $\alpha 1(I)$ chain of type I collagen in a patient with mild dominantly inherited osteogenesis imperfecta. *Mol Biol Med* 1988;5:197-207.
 - 23 Sillence DO, Senn A, Danks DM. Genetic heterogeneity in osteogenesis imperfecta. *J Med Genet* 1979;16:101-16.
 - 24 Nicholls AC, De Paape A, Narcisi P, et al. Linkage of a polymorphic marker for the type III collagen gene (COL3A1) to atypical autosomal dominant Ehlers-Danlos syndrome type IV in a large Belgian pedigree. *Hum Genet* 1988;78:276-81.
 - 25 Bonadio J, Byers PH. Subtle structural alterations in the chains of type I procollagen produce osteogenesis imperfecta type II. *Nature* 1988;336:363-6.
 - 26 Barsh GS, Peterson KE, Byers PH. Peptide mapping of collagen chains using CNBr cleavage of proteins within polyacrylamide gels. *Collagen Rel Res* 1981;1:543-8.
 - 27 Nicholls AC, Oliver J, Renouf DV, McPheat J, Palen A, Pope FM. Ehlers Danlos syndrome type VII: a single base change that causes exon skipping in the type I collagen $\alpha 2(I)$ chain. *Hum Genet* 1991;87:193-8.
 - 28 Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T₄. *Nature* 1970;227:680-5.
 - 29 Bonner WM, Laskey RA. A film detection method for tritium labelled proteins and nucleic acids in polyacrylamide gels. *Eur J Biochem* 1974;46:83-8.
 - 30 Maniatis T, Fritsch EF, Sambrook J. *Molecular cloning: a laboratory manual*. New York: Cold Spring Harbor Laboratories, 1982: 191-3.
 - 31 Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain terminating inhibitors *Proc Natl Acad Sci USA* 1977; 74:5436-67.