

Determination of a new collagen type I $\alpha 2$ gene point mutation which causes a Gly640 Cys substitution in osteogenesis imperfecta and prenatal diagnosis by DNA hybridisation

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Abstract

The molecular defect responsible for a sporadic case of extremely severe (type III/III) osteogenesis imperfecta was investigated. The mutation site was localised in the collagen type I $\text{pro}\alpha 2$ mRNA molecules produced by the proband's skin fibroblasts by chemical cleavage of mismatch in heteroduplex nucleic acids. Reverse transcription-polymerase chain reaction DNA amplification, followed by cloning and sequencing, showed heterozygosity for a G to T transversion in the first nucleotide of exon 37 of the COL1A2 gene, which led to a cysteine for glycine substitution at position 640 of the triple helical domain. This newly characterised mutation is localised in a domain which contains several milder mutations, confirming that glycine substitutions within the $\alpha 2(I)$ chain do not follow a linear gradient pattern for genotype to phenotype correlations. In a subsequent pregnancy, absence of the G2327T mutation in the fetus was shown by allele specific oligonucleotide hybridisation to the trophoblast derived fibroblast mRNA after reverse transcription and in vitro amplification. (The nucleotide number assigned to the mutant base was inferred from the numbering system devised by the Osteogenesis Imperfecta Analysis Consortium (The OIAC Newsletter, 1 April 1994).)

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Osteogenesis imperfecta (OI) is a heritable disorder of connective tissue associated with abnormalities of the structure or the synthesis of type I collagen.^{1,2} In most cases, heterozygosity for a point mutation at one of the two genes responsible for collagen type I synthesis, COL1A1 and COL1A2, has been shown, which results in the substitution of a glycine residue in collagen chains. Glycines are regularly repeated 338 times in the Gly-X-Y units of the collagen triple helical domain. Such substitutions have been associated with a wide range of clinical forms, from perinatal lethal to extremely mild, and are currently classified into four clinical types.³ The attempt to correlate genotype with phenotype has taken into ac-

count the position and the type of substituting amino acids, and it has proven more complex than expected. A "linear gradient" model has been proposed, particularly for cysteine substitutions along the $\alpha 1(I)$ chain, whereby the severity of the disease is directly proportional to the distance of the mutation from the amino-terminal end of the triple helix.⁴ This model, however, does not seem to apply for cysteine substitutions in the $\alpha 2(I)$ chain, where lethal and non-lethal mutations intermingle: the presence of discontinuous domains which contribute differently to helix stability has been inferred,⁵ and five alternating non-lethal-lethal regions have been proposed.⁶

Another open issue in OI research is genetic risk assessment for healthy parents who have had a type II (lethal perinatal) or type III (severe) OI infant: an empirical recurrence risk of 6 to 7% has been determined, owing to parental germline mosaicism for the mutation.⁷ Quantification of the mutant allele in the germline can be done easily in males: sperm DNA of mosaic fathers' ratios range from 12% to 40%, with the recurrence risk varying accordingly.⁸⁻¹⁰ In all cases documented so far for OI, mosaicism was also detected in leucocytes, although at different levels from spermatozoa. However, the possibility of finding isolated germline mosaicism cannot be excluded.

Even when molecular genetic studies of parental DNA are not feasible, once the causative mutation has been characterised in a type II (or type III) OI infant, recurrence in the same family can be determined by prenatal testing on chorionic villus cells.

Here we report a new COL1A2 mutation found in the first child of a healthy couple, and a prenatal diagnosis performed on the subsequent pregnancy, where recurrence of the mutation was excluded.

Materials and methods

CASE REPORT

The proband was the first child of healthy, non-consanguineous parents, aged 35 (mother) and 31 (father). The baby boy was born by caesarean section at term after an uneventful pregnancy. Birth weight was 2260 g. Apgar scores were 8 at one minute and 10 at five minutes. Skeletal x rays showed a skull with wormian bones and multiple fractures involving the ribs, radius and ulna on the right, the

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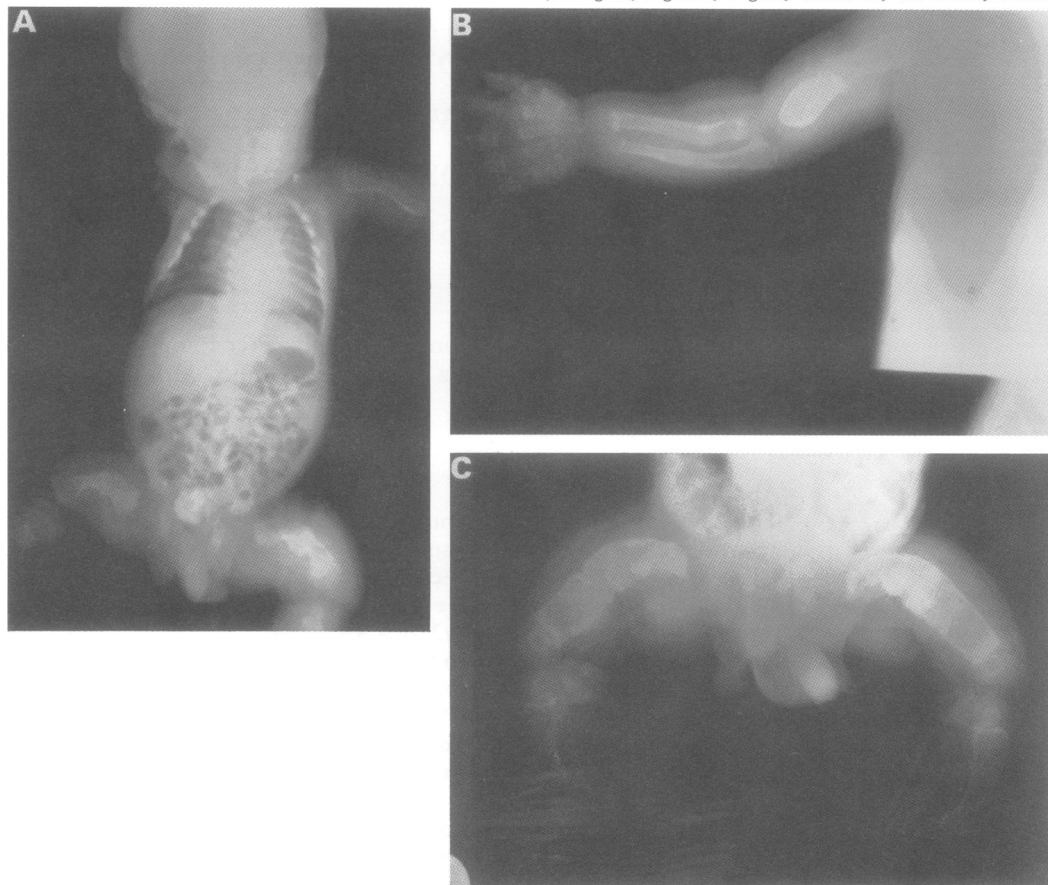


Figure 1 Total body radiograph of OI II infant showing multiple fractures of the ribs, upper and lower limbs (A), and detail of upper right (B) and both lower (C) limbs.

humerus and ulna on the left, and the lower limbs bilaterally. Vertebral bodies were flattened (fig 1).

On clinical examination the baby presented with prominent eyes with blue sclerae, a beaked nose, and shortening and bowing of the upper and lower limbs. He died at 6 months from cardiorespiratory failure. The clinical form was classified as extremely severe/lethal (type III/II).

LOCALISATION OF THE MUTATION BY CCM ANALYSIS

A skin biopsy from the proband and his parents was obtained after informed consent; dermal fibroblast cultures were established and maintained according to standard procedures. Total RNA was prepared from the patient's fibroblasts by the RNAsfast method (Molecular Systems). Approximately 5 µg of RNA were hybridised to $\alpha^{32}\text{P}$ -dCTP end labelled overlapping wild type cDNA probes, obtained from full length pro α 1(I) cDNA¹¹ and pro α 2(I) cDNA.¹² Hydroxylamine and piperidine treatment were as described previously.¹³

RT-PCR AND SEQUENCE DETERMINATION

First strand cDNA was synthesised from approximately 5 µg of total RNA with MMLV reverse transcriptase (BRL) and 100 pmol of the oligonucleotide primer 741R: 5' TTAGGCCCTTTGCCTCCTCT 3', complementary to sequences in exon 37 of the COL1A2 gene. One tenth of the reaction mix-

ture was used for polymerase chain reaction (PCR) amplification; the second PCR primer was 662F: 5' CAGAGGTGAAATTGGTAAC 3', corresponding to sequences in exon 33. Amplification conditions were: 93°C for 30 seconds, 54°C for 30 seconds, 72°C for 30 seconds, for 30 cycles, using AmpliTaq polymerase (Perkin Elmer-Roche). The 256 bp PCR product was cloned into a pCR-Script SK(+) vector (Stratagene). Five clones were sequenced by the dideoxy chain termination method using an Auto Read Sequencing kit and ALF DNA Sequencer from Pharmacia-LKB.

MUTATION DETECTION BY ALLELE SPECIFIC OLIGONUCLEOTIDE HYBRIDISATION

PCR products obtained, as described above, from various RNAs, were dot blotted on Hybond C (Amersham) duplicate filters. Oligonucleotides carrying either the normal (N) 5' TCCTGCTGGTGCTGCTGGT 3' or the mutant (M) 5' TCCTGCTTGTGCTGCTGGT 3' sequence, were end labelled with $\gamma^{32}\text{P}$ ATP and T₄ polynucleotide kinase (Pharmacia). Hybridisation was carried out at 52°C. Filters were rinsed once at room temperature with 2 × SSC, 0.1% SDS, then at 59°C for five minutes in the same buffer.

Results

Analysis of pepsin treated collagens in the proband had shown the presence of both normal and abnormal chains with delayed electrophoretic

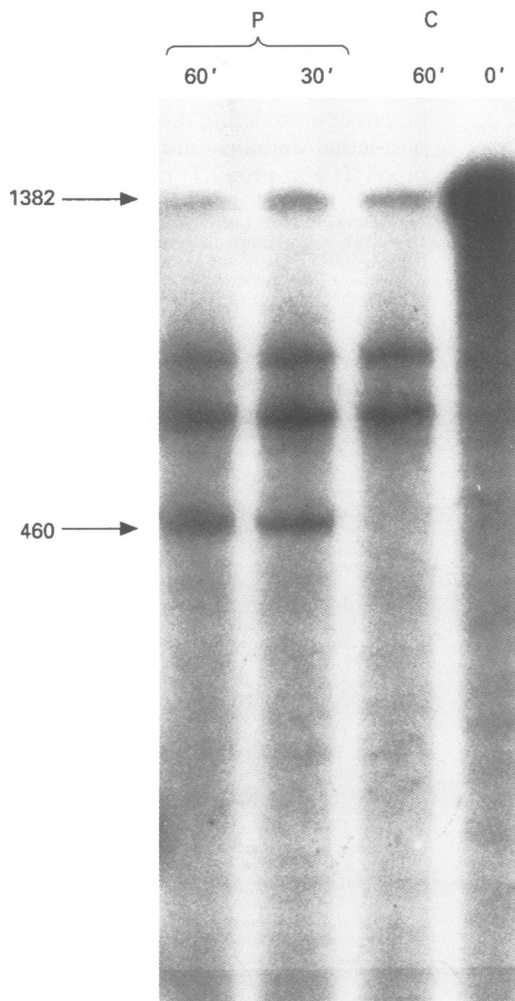


Figure 2 CCM analysis of heteroduplexes formed between patient's (P) and control (C) RNA and a 1382 bp *NcoI-NcoI* $\alpha 2(I)$ cDNA probe. Incubation with hydroxylamine was for the times indicated. The samples were subjected to electrophoresis on a 6% acrylamide/5 mol/l urea gel and autoradiography. The arrow points to the mismatch specific fragment product (460 bp). MW standard (not shown) was *HaeIII*- Φ X174 DNA.

mobility, which were secreted efficiently by dermal fibroblasts (data not shown). In order to localise the mutation, $\text{pro}\alpha 1(I)$ and $\text{pro}\alpha 2(I)$ mRNAs were screened by the chemical cleavage method (CCM).¹⁴ Heteroduplexes formed with a 1382 bp *NcoI-NcoI* $\text{pro}\alpha 2(I)$ probe showed a mismatch specific band on incubation with hydroxylamine followed by piperidine treatment (fig 2). A 256 bp region of cDNA, encompassing the putative mutation site, was generated by reverse transcription-amplification (RT-PCR) and cloned into a pCR-Script SK(+) vector (Stratagene). Five clones were sequenced: three of them had a G to T transversion at the first nucleotide of exon 37 in the $\text{pro}\alpha 2(I)$ coding sequence, which caused a G640C substitution (the first glycine of the triple helix is referred to as position 1) (fig 3).

In the subsequent pregnancy, a prenatal test for the diagnosis of disease recurrence was requested by the couple. Allele specific oligonucleotide (ASO) 19mers for mutation 640C and for the corresponding normal sequence were synthesised and radiolabelled.

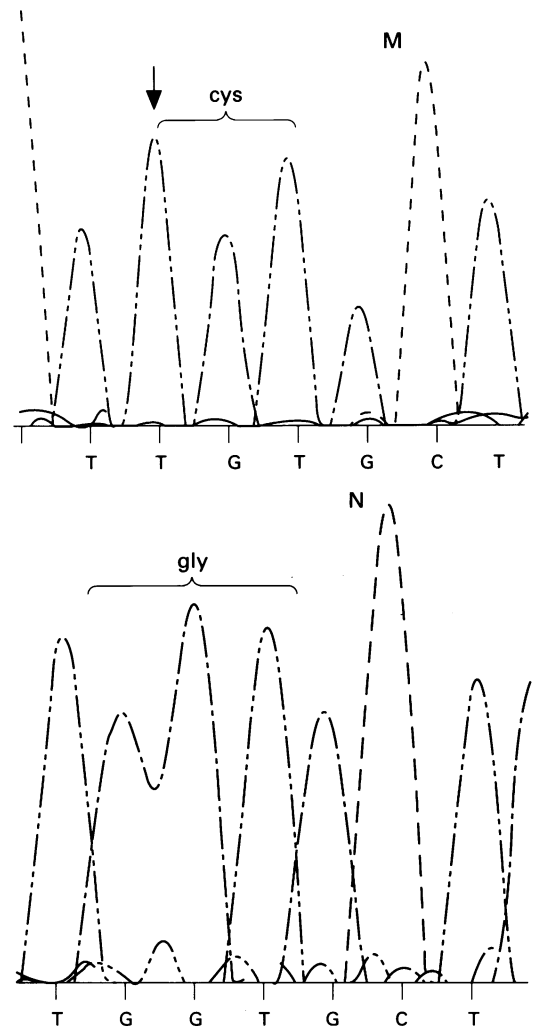


Figure 3 Sequence determination (*ALF DNA Sequencer-Pharmacia*) of the mutant (M) and the normal (N) *COL1A2* alleles of the patient. The arrow indicates the G-T transversion.

Mutation detection was achieved by ASO hybridisation on RT-PCR products from fibroblasts obtained by in vitro culture of the 10th week chorionic villus sample. Detection at the genomic level was not feasible, since the mutated base is the first nucleotide of exon 37, and we did not have any sequence information on the preceding intron. Fig 4 shows that, while heterozygosity was confirmed in the OI baby, the mutation was not found in the fetus, nor in the parents' fibroblasts, therefore excluding recurrence of the disease. The birth of a healthy boy has recently confirmed the molecular diagnosis.

Discussion

Our results provide molecular evidence for a new *COL1A2* mutation, G640C, which caused lethal OI. The glycine substitution described here maps closely to a previously described G646C substitution, which causes mild/moderate familial OI.⁵ Our observation therefore confirms that for cysteine substitutions in

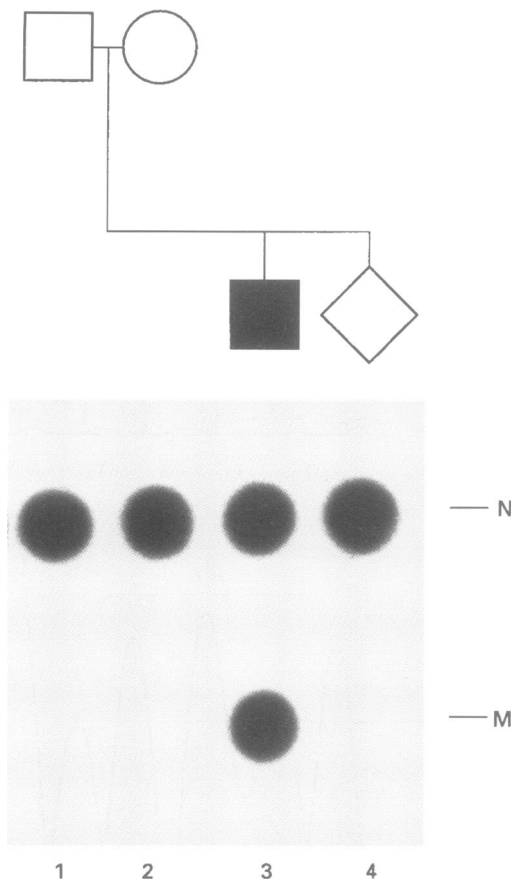


Figure 4 Pedigree of the family studied and mutation detection by allele specific oligonucleotide hybridisation on RT-PCR amplified cDNA from parental fibroblasts (1, 2), OI fibroblasts (3), and the chorionic villus cells (4). Duplicate filters were hybridised either to the normal (N) or to the mutant (M) allele specific oligonucleotide, as described in Materials and methods.

pro α 2(I) chains, there is no simple relationship between phenotype and distance of the substituting residue from the NH₂-terminus.¹⁵ The residues immediately adjacent to the mutation might play a critical role in influencing the triple helix stability. Furthermore, the lethal mutation reported here maps within a region between residues 586 and 676 of the α 2(I) chain where four non-lethal and no lethal glycine substitutions have been mapped so far. For this reason it has been proposed as a

“tolerant”, that is, non-lethal, microdomain.⁶

With the localisation of additional glycine substitutions along the COL1A2 gene and the gradual saturation of the mutation map, it will be possible to define more precisely lethal and non-lethal domains and microdomains within pro α 1(I) and pro α 2(I) polypeptide chains.

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