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Genetic counselling on brittle grounds: recurring osteogenesis imperfecta due to parental mosaicism for a dominant mutation

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Abstract Osteogenesis imperfecta (OI), a dominantly inherited connective tissue disorder, is usually caused by defects in collagen I. There is growing evidence for parental mosaicism that results in affected children born to unaffected parents. This situation poses a difficult task for the geneticist because a mosaic parent may appear clinically healthy while carrying the mutation in a fraction of her or his gonadal cells. To illustrate this problem, we report a Swiss couple whose first child was affected with severe OI. The unexpected recurrence of the disorder in the second child raised the suspicion of a recessive trait or, rather, of parental mosaicism. We identified the responsible collagen mutation in the COL1A2 gene (Gly688Ser in the α2(I)-chain) in both children and demonstrated the father to be a somatic mosaic for this mutation and to have subtle clinical signs such as soft skin and short stature that may be a result of his mosaic state.

Conclusion After the birth of a child affected with OI the possibility of parental mosaicism should be considered and options for prenatal diagnosis discussed.

Key words Osteogenesis imperfecta
Collagen I · Mosaicism · Genetics
Recurrence risk

Abbreviations *OI* osteogenesis imperfecta · *PCR* polymerase chain reaction · *SSCP* single strand conformation polymorphism

Introduction

Osteogenesis imperfecta (OI) – brittle bone disease – is a heritable, generalised connective tissue disorder characterised by fragile bones and weakness of other tissues rich in collagen I [3]. In the vast majority of cases, mutations have been found that alter the primary structure of the α1(I)- or α2(I)-chains of collagen I. The most common mutations found to date have been single-base alterations that substitute amino acids with bulkier side-chains for the glycine required in every third position of the chain [reviewed in 3]. Only a few OI patients without defects in collagen I have been reported [24] with an apparent autosomal recessive mode of inheritance.

It was once thought that the mode of inheritance of OI could be separated into autosomal dominant and autosomal recessive traits [19]. This opinion was challenged when two groups reported that the risk of recurrence of severe and lethal OI (types III and II, respectively) in clinically healthy couples was 5%–8%, and thus significantly below that of the recurrence risk of 25% for recessive disorders [3, 22]. However, this observed risk appeared too high to be compatible with repeated spontaneous new mutations in every affected child following the index case. It was speculated that a parent, due to a mutation in one cell during her or his early ontogeny, might carry the mutation in some, but not all somatic and gonadal cells and therefore represent a mosaic status. Depending on the proportion and tissue distribution of the mutation, the mosaic

parent therefore would appear clinically as only mildly, if at all, affected.

In fact, such a mechanism for the inheritance of identified collagen I mutations causing OI has been reported [2, 6, 7, 9, 14–16, 23]. The eight mutations are all different; six of them reside in the α1(I)-chain, the remaining two in the α2(I)-chain [9, 14]. Since it is becoming clear that these few incidences represent only the tip of the iceberg, we want to call the possibility of parental mosaicism in OI to the attention of the genetic counsellor. To illustrate this problem, we report a clinically healthy couple whose first child was affected with severe OI. The recurrence risk was estimated to be very low, because of the low likelihood of *de novo* occurrence of the same or another new mutation and because recessive inheritance was also regarded as improbable. However, the second child was also affected with severe OI and the recurrence of the disorder was due to the same point mutation in the COL1A2 gene (Gly688Ser) caused by paternal mosaicism in the somatic and gonadal line.

Case reports

The family history of this non-consanguineous Swiss couple was unremarkable with regard to connective tissue disorders; there was no significant medical history of fractures due to minor trauma, joint hypermobility, scleral discolouration, bone deformities, or dental abnormalities. Both parents were 29 years old when their first child, which was affected, was born. The father measured 162 cm and was shorter than his two other brothers (170 cm and 172 cm). On physical examination his skin was remarkably thin and soft. He had loose knee joints and ankles and wore supporting shoes. The mother's height of 162 cm was within the range of that of her two sisters (160 cm and 157 cm); she had five brothers, three of whom were taller than her. On physical examination her skin and the joint mobility were normal. A radiological skeletal survey was normal in both parents.

Case 1 (A.H., 6.8.1979), their first child was born 10 days before term (weight 2800 g, length 46.5 cm) in a peripheral hospital. OI was diagnosed at age 18 days, when the first X-ray examination revealed a fresh longitudinal left femoral fracture, multiple older fractures and Wormian bones. By the age of 12 years she had suffered approximately 75 fractures due to minor traumata and had markedly deformed bones. The initial dark blue hue of her sclerae had faded to a greyish tint. Clinically, her deciduous teeth were opalescent, whereas her permanent teeth were large, white and appeared normal, but radiographically showed narrowing of the pulp chamber and pulp stones. She heard well. Up to the age of 3 years she could walk with assistance but generally preferred to crawl on the floor because of pain in her spine. Deformation of her femoral bones progressed rapidly as did severe osteopenia which led to compressed lumbar and thoracic vertebral bodies. Thus, she never regained ambulatory status and was wheel-chair bound. Up to the age of 9 months, both her weight and length were between the 3rd and 10th percentiles. Since then, her weight has always been at least 10 kg below the 3rd percentile (her weight at the age of 14.5 years was 17 kg). Her head circumference ranged between the 10th and 25th percentile.

The parents sought genetic counselling in 1980. They were told that the cause for their daughter's disorder was most likely a spontaneous dominant mutation with a very low recurrence risk, or, less likely, a recessive trait with a recurrence risk of 25%. After an uneventful pregnancy, the second child (case 2; T.H., 5.8.1981) was delivered by elective Caesarean section 14 days before term (weight 2920 g, length 44 cm) in the same peripheral hospital. As a newborn, he had a right inguinal hernia. OI was diagnosed only at 6 months after a fresh humerus fracture. X-ray examinations on this occasion revealed a few older fractures and Wormian bones, albeit less prominent than in his sister. By the age of 10 years he had sustained about 30 fractures. His deciduous teeth were opalescent, whereas his permanent teeth were white and large, but showed radiographically narrowing of the pulp chambers and pulp stones; there was no impairment of hearing. He differed from his sister in that he had an apparent later onset of symptoms, had only light blue sclerae, fewer fractures, less severe bone deformities and less osteoporosis; as a result of that he has always been significantly taller than his sister. Weight and length remained slightly below the 3rd percentile. His present weight at age of 12.5 years is 28 kg. His head circumference has been following the 10th percentile. He was able to walk with aids until 1992, but lost ambulatory status when a series of fractures aggravated deformation of both proximal femora. He is presently wheel-chair bound.

In 1991 we offered biochemical and molecular investigation to the family. With informed consent, we obtained skin punch biopsies from all family members and established dermal fibroblast cultures. In addition, EDTA-blood, saliva and hairbulbs were collected from both parents and stored at –20°C.

Materials and methods

Biochemical analysis of cell cultures

Dermal fibroblasts were cultured in MEM with 10% FCS (both Gibco) and grown under standard conditions. Cells were seeded (250 000 per 35 mm dish) and after 24 h were metabolically labelled for another 16 h with 10 μCi each of [^{3}H]proline and [^{3}H]glycine in the presence of 50 μg/ml ascorbate and 50 μg/ml catalase. Separate harvesting and pepsinisation of collagen from culture medium and cell layer were as described [20]. After electrophoresis on 5% polyacrylamide gels in the presence of 0.5 M urea the radiolabelled collagens were detected by autoradiofluorography. Two-dimensional mapping of CNBr-cleavage peptides of collagen chains (cleavage time 90 min) was performed as described [20].

Thermal stability of triple helical collagen

Fibroblasts were cultured and metabolically labelled under standard conditions [20]. Collagen secreted into the medium was treated with pepsin, and thermal stability and the melting profile of triple helical collagen were determined as described [21]. Briefly, a solution of collagen was gradually heated (12.0°C/h), aliquots were tested at 0.5°C intervals for triple helicity with trypsin (10 μg/ml final concentration) and the proteins were separated by electrophoresis. Melting temperature (T_m) of normal collagen and abnormal collagen, as judged by electrophoretic retardation, was defined as the temperature at which 50% of the normal or abnormal α(I)-chains had disappeared.

Reverse transcriptase polymerase chain reaction (PCR) amplification, single strand conformation polymorphism (SSCP) analysis and DNA sequence analysis

Total cellular RNA was prepared from cultured fibroblasts and cDNA was synthesised from this, using random hexadeoxyribonucleotides and an established method [11]. Primers SSCP1A2γL (5′ GCCGGTCCTACTGGTCCTAT 3′) and SSCP1A2γR (5′

CACCACGGTTTCCATGTTTG 3′) were used to PCR amplify a 1141 bp fragment of the proα2(I) mRNA [12]. Reactions were cycled 34 times at 95°C for 1.4 min, 66°C for 1 min and 70°C for 1.4 min. SSCP analysis was carried out as described [13] after digestion of PCR products labelled by incorporation of [α-^{32}P]dCTP. A 504 bp MaeII fragment encompassing codons for residues 624 to 792 (numbering the first glycine of the triple-helical region as 1) was subcloned into ClaI-digested, phosphatase-treated pBluescript SK- [18]. Sequence analysis was performed using T7 DNA polymerase with the method recommended by Pharmacia.

Genomic PCR and allele-specific oligonucleotide hybridisation

Genomic DNA was prepared from cultured fibroblasts, hair root bulbs, buccal cells and white blood cells using standard procedures [17]. Approximately 200 ng DNA was used as template for amplification with the primers 1A2-HFL (5′ CTTCTAAGAGATGCGGGAAT 3′) and 1A2-HFR (5′ CTGATAGCAACATACACTGA 3′) [12]. Reactions were cycled 32–35 times at 95°C for 1 min, 56°C for 1 min and 70°C for 1 min. DNA concentrations were standardised by agarose gel electrophoresis and dilutions of DNA in volumes of 7.5 µl were denatured by addition of 50 µl denaturing solution (43 µl 10 mM Tris/0.5 mM ethylenediamine tetraacetic acid, pH 7.5; 4 µl 6 M sodium hydroxide; 3 µl 0.5 ethylenediamine tetraacetic acid, pH 7.5) [25]. After 10 min incubation on ice, denatured DNA was transferred under vacuum to Hybond-N+ filters, with the use of a Hybrislot Manifold (Gibco BRL). After loading the DNA, each well was rinsed with a further 50 µl of denaturing solution and the filter was rinsed in 3×SSC (1×SSC is 0.015 M trisodium citrate (pH 7.0), 0.15 M NaCl). Allele-specific oligonucleotide probes used were ASO wt (5′ GGAAGTCGTGGTGATGGAGG 3′) for detection of the normal allele and ASO mut (5′ GGAAGTCGTAGTGATGGAGG 3′) for detection of the mutant allele. Oligonucleotides were end-labelled with [γ-^{32}P]dATP [17].

Filters were prehybridised for at least 6 h in 6SSC, 5×Denhardt solution (0.1% (w/v) Ficoll 400, 0.1% (w/v) polyvinyl pyrrolidone and 0.1% (w/v) bovine albumin, fraction V), 0.5% sodium dodecyl sulphate and 100 mg/ml denatured sheared salmon sperm DNA at 62.5°C, then were transferred to fresh solution containing labelled probe. Hybridisation was allowed to proceed overnight at 62.5°C. Filters were washed in 6×SSC at 61.0°C for 10 min, followed by a 10 min wash at 62.5°C. Filters were autoradiographed and, if necessary, any further non-specific signal was removed by washing at progressively higher temperatures. After hybridisation of filters to ASO mut, the probe was stripped and filters were then hybridised to ASO wt using the same hybridisation and wash conditions.

Quantification of the mutant allele was performed by blotting DNA onto a filter alongside known dilutions of DNA from case 1 (assumed to contain 50% mutant sequence) with DNA from a normal individual. Intensities of signals were compared visually after autoradiography. Exposures of filters probed with ASO mut and ASO wt were standardised by comparison of signals from OI patient case 1.

Results

Biochemical findings

Collagen analysis in fibroblasts established from both patients showed impaired secretion and increased retention of collagen I. There was retarded electrophoretic mobility of both α1(I)- and α2(I)-chains of collagen I retained in the cell layer and secreted into the culture medium (Fig. 1).

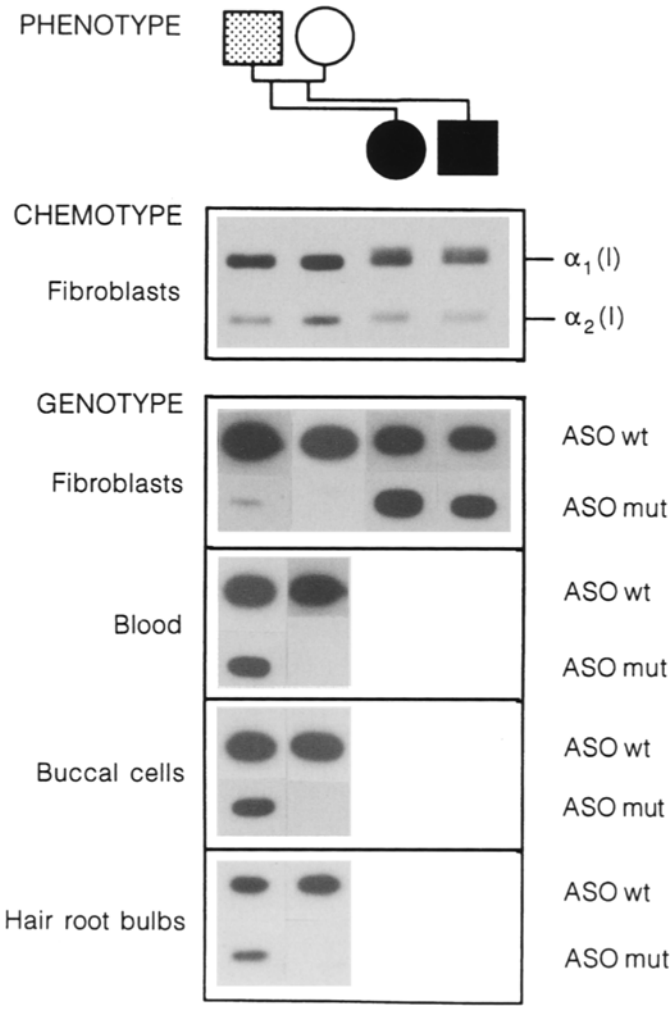

Fig. 1 Comprehensive illustration of phenotype, chemotype and genotype of the family. *Phenotype*: both affected children are indicated by *closed symbols*, the oligosymptomatic mosaic father by a *stippled symbol*, and the unaffected mother by an *open symbol*. *Chemotype*: fibroblast cultures of each family member were incubated with [^{3}H]proline and [^{3}H]glycine, and metabolically labelled collagen from the culture medium was pepsinised and analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis. Both affected children produced a population of collagen α1(I)- and α2(I)-chains with markedly retarded electrophoretic mobility; collagen from the mother's cells migrated normally. In the depicted experiment cells of passage 2 from the father were examined; there are subtle signs of electrophoretic retardation which was absent in later passages. *Genotype:* allele-specific oligonucleotide (ASO) hybridisation results from DNA cell types from the family. DNA from the affected siblings hybridised to normal wild type sequence (ASO wt) or mutant sequence (ASO mut) in similar proportions. ASO mut hybridises with DNA from different cell types from the mosaic father in varying ratios relative to ASO wt, whereas there is no hybridisation of ASO mut with DNA extracted from the various cell types of the mother

Two-dimensional CNBr-peptide mapping localised the region of overmodification to the CB7 peptide of the α1(I)-chain or the corresponding region on the α2(I)-chain in

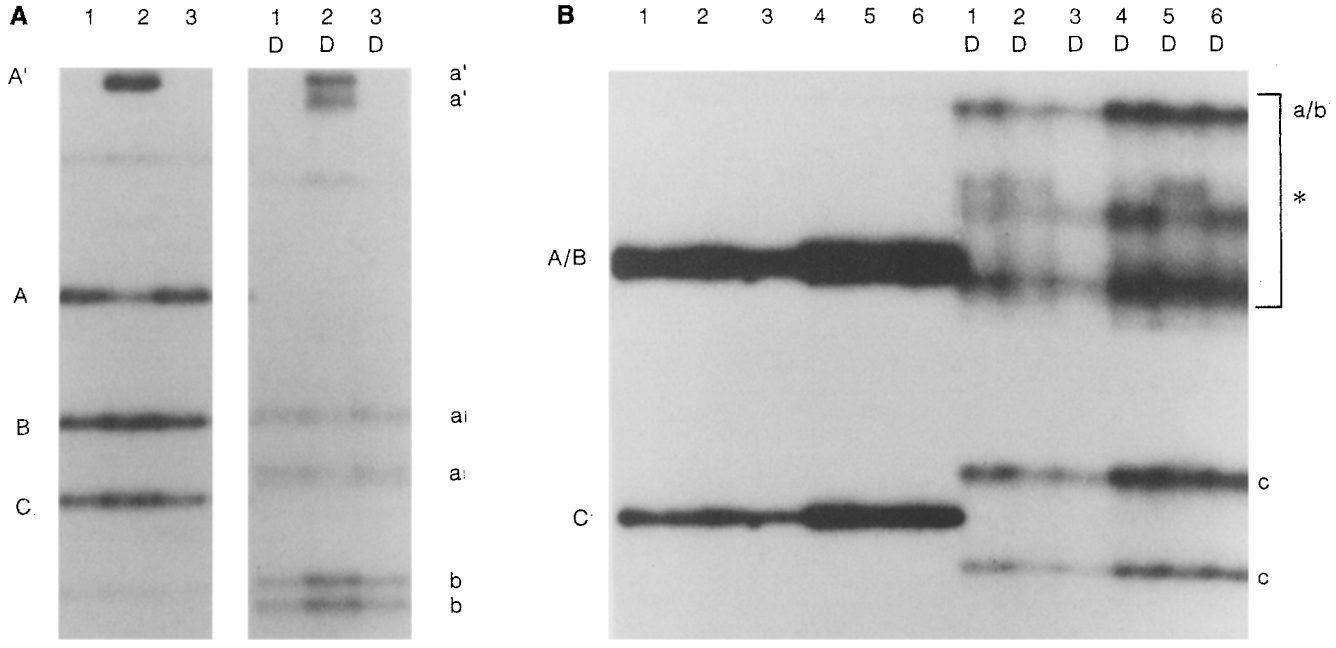

Fig. 2 SSCP analysis of *Hph*I and *Dde*I digests. Non-denatured samples are shown on the left and denatured samples (indicated by *D*) on the right. Double-stranded fragments are marked by *upper-case letters* and presumed corresponding single-stranded fragments are marked with *lower-case letters*. Not all fragments are shown. **A** Lane 2- DNA from case 1, lanes 1 and 3- DNA from controls. Fragments resulting from loss of an *Hph*I recognition sequence are indicated by A' and a'. **B** Lanes 1- DNA from case 2, lanes 2 and 5-DNA from case 1, lane 3- DNA from mother, lane 4- DNA from father, lane 6- DNA from control. The band-shift is indicated by an *asterisk*

collagen I from both children (not shown). No abnormalities were found in the parents. The melting profiles of collagen I from culture medium of cells of both affected children were identical and showed a decreased melting temperature of 40.5°C for abnormal collagen I containing a mutant α2(I)-chain and a normal melting temperature of 41.5°C for normal collagen I.

Identification of the mutation

SSCP analysis was carried out using RNA from case 1 in an attempt to identify the causative mutation. Four pairs of primers have previously been designed to allow PCR amplification of the majority of proα1(I) mRNA sequence and subsequent screening for mutations by SSCP analysis after restriction enzyme digestion [13]. Two of these primer pairs are required to amplify the region of α1(I) mRNA coding for α1(I) CB7 peptide. No indication of a mutation was found on screening of these fragments. Because of the evidence that the mutation was within the region encoding α1(I)CB7, the corresponding region of the proα2(I) mRNA was analysed. Primers SSCP1A2γL and SSCP1A2γR amplify a 1141 bp fragment of the proα2(I) mRNA which encompasses the region corresponding to α1(I)CB7.

*Dde*I, *Alu*I/*Sty*I, *Msp*I, *Hph*I and *Nci*I digests of the 1141 bp PCR product amplified from case 1 and two controls were analysed. The resulting fragments were identical in the control samples. However, in case 1, *Hph*I digestion revealed a decreased intensity of the largest *Hph*I fragment, 198 bp in length, with the appearance of a larger fragment (Fig. 2A). This was consistent with a mutation resulting in the loss of an *Hph*I recognition site. A band-shift was observed in the largest band in the *Dde*I digest (e.g. see Fig. 2B). *Dde*I digestion of the PCR product produces fragments of 351, 348, 253 and 189 bp, but the two largest fragments were not resolved and ran as a single band. Therefore, it was not known which fragment gave rise to the band-shift. However, the largest *Hph*I fragment was contained within the 351 bp *Dde*I fragment (Fig. 2A) and therefore it was assumed that the band-shift arose from this fragment.

SSCP analysis of *Dde*I digests was repeated using material PCR amplified from RNA from the family members. Both parents showed the same pattern of bands as the normal control, whereas both offspring, case 1 and case 2, showed the extra band (Fig. 2B). Also, only the two affected siblings, and not the parents, showed loss of the *Hph*I site described (data not shown). Thus, it was likely that the *Dde*I band-shift and loss of the *Hph*I site resulted from the mutation which gave the OI phenotype.

Figure 3A shows restriction maps of the PCR product and the data from SSCP analysis. The 351 bp *Dde*I fragment contains two *Hph*I sites which, if disrupted, could produce the size of polymorphic band observed. A *Mae*II digestion fragment was subcloned from DNA from both

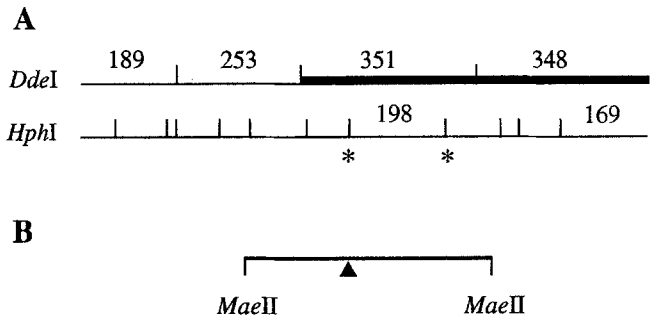

Fig. 3 A *Dde*I and *Hph*I restriction maps of the 1141 bp fragments used in SSCP analysis. *Numbers* indicate sizes, in bp, of relevant fragments. The *bold lines* indicate the two fragments which could have given rise to the band-shift in SSCP analysis. *Asterisks* on the *Hph*I map indicate the recognition sites which could harbour the mutation. **B** The *Mae*II fragment subcloned for sequence analysis is indicated and the position of the G to A mutation identified is shown by an *arrowhead*

case 1 and case 2, and six subclones from each were sequenced, using the appropriate M13 primers after determining the orientation of insert. Of these, five subclones from case 1 and three subclones from case 2 contained a G to A transition within the *Hph*I site situated towards the 5′ end of the gene (Figs. 3B, 4), whereas the remaining subclones had a G. The mutation changed a glycine codon, GGT to a codon for serine, AGT. Thus, a heterozygous G to A transition, causing the substitution of α2(I) glycine-688 with a serine, was found in both offspring affected with OI. This residue is within the region of the α2(I) mRNA corresponding to α1(I)CB7 and is therefore consistent with the pattern of overmodification of CB peptides.

Somatic mosaicism in the father

The mutation had not been detected in the parents' fibroblast RNA by SSCP analysis. Therefore, an approximately 400 bp fragment of COL1A2 was amplified, using primers 1A2-HFL and 1A2-HFR, from genomic DNA prepared from blood cells, buccal cells, fibroblasts and hair root bulbs. DNA was then used in allele-specific oligonucleotide hybridisations, which indicated a relatively high level of mosaicism in buccal and blood cells, and a low level in fibroblasts and hair roots from the father but not from the mother (Fig. 1). Approximately 30%–40% of cells in blood and saliva, 12% of hair root bulb cells and 3% of fibroblasts carried the G to A transition.

Discussion

Mosaicism can be an important and sometimes dramatic cause of phenotypic variation in the expression of genetic traits. Germline mosaicism can lead to familial clustering of affected individuals and provides an explanation for the

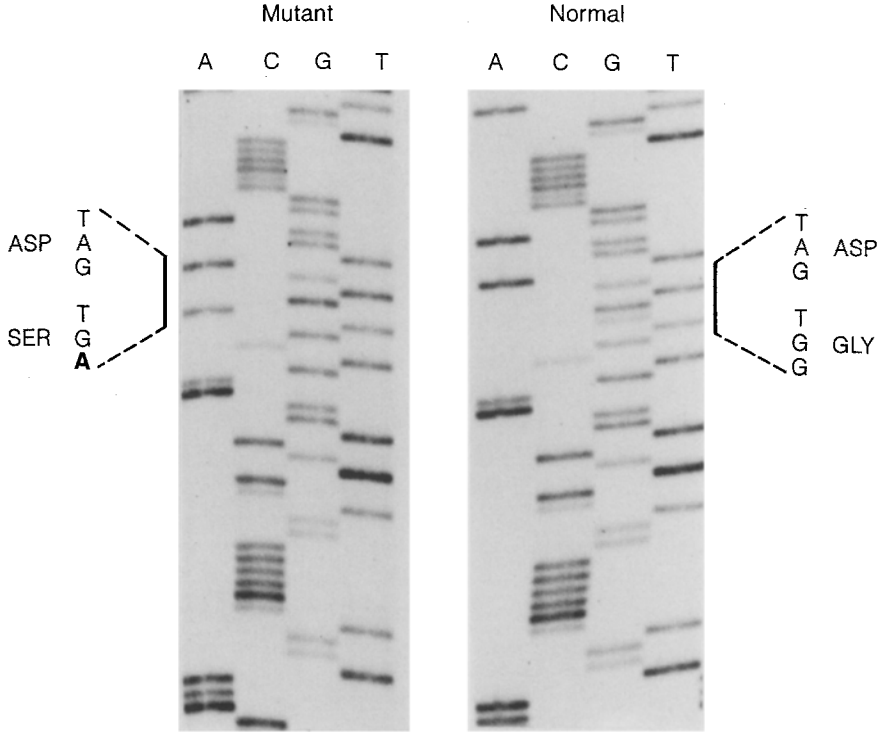

Fig. 4 Sequence of the sense DNA strand from subclones showing mutant and normal sequence. The mutant base is shown in *bold type* and the amino acids encoded are indicated

recurrence of rare dominant mutations within a single family. This has been described in a variety of conditions [10], such as Duchenne muscular dystrophy, achondroplasia, haemophilia A, Apert syndrome, and Crouzon craniofacial dysostosis, amongst which osteogenesis imperfecta has become a prominent example. Including this communication, a total of nine identified cases of somatic or gonadal mosaicisms has been reported in OI [2, 6, 7, 9, 14–16, 23] but there are other cases of parental mosaicism where the exact mutation has not been identified yet [1, 5, 8; Peter Byers, personal communication, September 1993]. In the light of these findings, we have reason to strongly suspect that the identified mosaic cases represent only the tip of the iceberg.

The usual source for biochemical collagen analysis are dermal fibroblast cultures established from skin biopsies. In the case of mosaicism for an OI mutation restricted to gonadal cells, no biochemical evidence for a collagen defect would be detectable in fibroblasts. In somatic mosaicism, however, identification of defective molecules will depend on the proportion of heterozygous cells present in the respective tissue. The presence of well-compensated mosaic individuals points towards negative selection of heterozygous cell clones in vivo in tissues such as bone, tendon, skin, where collagen I is an essential component, but apparently not for blood and buccal cells. Evidence for negative selection of heterozygous fibroblasts in vitro came from the observation that overmodified collagen populations in early passages of a mosaic parent disappeared with increasing passage number [5, 8]. We have encountered the same phenomenon with fibroblast cultures from the mosaic father of the family reported here. During the first analyses, we were uncertain whether there were α(I)-chains with retarded migration. When we repeated the analyses with the same cells cultured for several passages the collagen chains migrated normally. Direct comparison of collagen produced by cells of an early and later passage showed subtle electrophoretic differences indicating a progressive loss of the cell population heterozygous for the mutation.

These studies suggest that the genetic counsellor needs to be aware that if a parent with mild OI has a child with a severe or lethal form, somatic mosaicism in the apparently mildly affected parent may be the explanation [5, 8, 9]. Furthermore, birth of an affected child to apparently normal parents needs to raise concern for parental mosaicism. It should be kept in mind how well "compensated" these mosaic parents can be, even despite a high level of mosaicism in cultured fibroblasts [9]. In mosaic individuals with a low proportion of cells heterozygous for the mutation, clinical identification can be very difficult. When we re-evaluated the clinical data of the family after the molecular characteriziation had been completed, we realized that we already had picked up subtle clinical hints for a mosaic status in the father, i.e. shorter stature than his male first-degree relatives, thin soft skin, and loose joints. In a similar case we had correctly predicted the mother to be mosaic because she was significantly shorter than her female first-degree relatives [16]. Remarkably, in both cases the search for radiological signs for OI (Wormian bones, osteopenia and deformation of vertebral bodies) was negative. We therefore believe that the pivotal medical skills – good history taking and careful physical examination – may help to uncover individuals mosaic for OI mutations.

We conclude that after the birth of a child affected with OI to a clinically healthy couple or of a severely affected child to a couple with one mildly affected partner, the genetic counsellor, ideally, should undertake all of the following measures: (1) to characterise the collagen defect of the index case at the protein and DNA level; (2) to try to clinically identify the possible mosaic parent to refine the average empirical risk of 7%–8% for recurrence of OI; meticulous history taking and physical examination may draw the attention to a parent with probable mosaicism, the demonstration of carrier status will identify him or her; (3) to offer DNA-based diagnostic studies on chorionic villus biopsies in future pregnancies; however, if the mutation is unknown, there is the useful alternative of rapid biochemical collagen analysis of chorionic villus organ culture allowing diagnosis within 1 week after sampling [16]. However, if none of the above options for prenatal diagnosis is available, ultrasonography is useful to detect OI although its sensitivity depends on the severity of OI, the gestational age and the examiner's expertise [4].

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