

Osteogenesis imperfecta Phenotypes Resulting from Serine for Glycine Substitutions in the $\alpha 2(I)$ Collagen Chain

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Key Words

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Abstract

Clinical and biochemical findings in 5 unrelated patients with osteogenesis imperfecta (OI) with a serine for glycine substitution in the $\alpha 2(I)$ collagen chain are presented. The data are compared to other serine substitutions in collagen type I. Findings show that the phenotypic severity of serine for glycine substitutions in the $\alpha 2(I)$ collagen chain is region dependent similar to the observations for the $\alpha 1(I)$ collagen chain, and that so-called 'lethal' and 'non-lethal' domains in the $\alpha 1$ and $\alpha 2$ collagen chains do not necessarily correspond.

Introduction

Osteogenesis imperfecta (OI) is a heterogeneous connective tissue disorder characterized by brittle bones. Four clinical subtypes are recognized, including a mild (type I), a lethal (type II), a severely deforming (type III) and a moderately severe (type IV) OI subtype [1]. A wide range of underlying mutations in COL1A1 and COL1A2 – the genes encoding both type I collagen α -chains – are known. These comprise glycine substitutions, splicing mutations causing exon skipping and, more rarely, large gene deletions [2].

The phenotypic outcome of glycine substitutions in type I collagen is influenced by the position and the nature of the substituting residue [2, 3]. It has been shown previously that glycine substitutions in the $\alpha 1(I)$ collagen chain generally show a linear gradient of phenotypic severity from the carboxyl- to the amino-terminal end of the molecule. However, as an increasing number of glycine substitutions in type I collagen is identified, more and more exceptions to this model are encountered. In this paper, we report 5 unrelated OI probands with gly-

cine to serine substitutions in the $\alpha 2(I)$ collagen chain and compare the phenotypic outcome with that of other serine substitutions reported to the Osteogenesis Imperfecta Mutation Consortium.

Materials and Methods

Clinical Summaries

Proband AU (fig. 1A) is a male fetus, second child of healthy, non-consanguineous parents. The pregnancy was terminated at 21 weeks of gestation after detection of multiple fractures on ultrasound. One elder sib is healthy. Clinical examination revealed a very small baby with a vertex to sacrum length of 16.6 cm and a weight of 390 g. There was shortening of the upper and lower limbs, marked bowing and deformity of the lower legs and abduction of the thighs.

Radiological features showed a thin, poorly ossified calvarium, thin ribs with no visible fractures, and some platyspondyly of the vertebrae. The femora were short and irregular, the tibiae broad and angulated, the fibulae very thin and angulated. Several fractures of the humeri and ulnae were seen. The clinical and radiological features were those of OI type IIB/III.

Proband PT (fig. 1B) is the first child of healthy, non-consanguineous parents, born at 40 weeks gestation after a breech delivery. Birth length was below the 3rd percentile. At birth, she showed sever-

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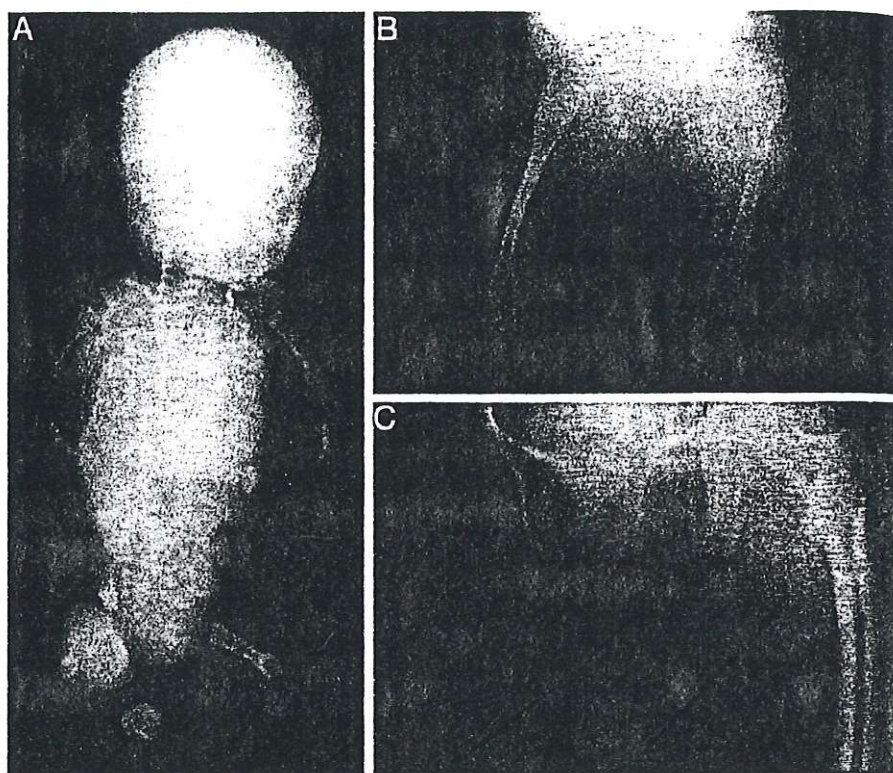
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Fig. 1. **A** Babygram of patient AU with the $\alpha 2(I)$ -G859S substitution showing poor ossification of the calvarium, thin ribs, mild vertebral platyspondyly, severe shortening of the femora and angulation of tibiae and fibulae. **B** X-ray of patient PT with the $\alpha 2(I)$ -G811S substitution, showing osteopenia, mild bowing of the femora and some widening of the metaphyses. **C** X-ray of patient WN with the $\alpha 2(I)$ -G682S substitution showing osteopenia and bowing of the femora.



al fractures and presented with a large head, triangular face, small nose, moderate hypotonia, enlarged fontanelles and white sclerae. Radiological features included relatively adequate ossification of the calvarium and multiple wormian bones, small, thin ribs and some platyspondyly of the cervical and thoracic vertebrae. The long bones were short but adequately modelled; there was some bowing and metaphyseal flaring of the humeri and tibiae. The femora were short and bowed, the fibulae were very thin. The clinical and radiological data fitted with OI type IV.

The proband's mother and one maternal uncle have multiple tooth caries and dentinogenesis imperfecta. One cousin has short stature and suffered several fractures. Two cousins of the proband's mother were diagnosed with Camurati-Engelman disease.

Proband WN (fig. 1C) is the second child of non-consanguineous healthy parents, born after an uneventful pregnancy. One older brother is healthy. A second-degree cousin is deaf-mute. The patient suffered her first fracture (right femur) at age 2½ years, the second fracture (left tibia) at age 8. Between age 8 and 10 years, she suffered 4 additional fractures of the right femur. She could not walk for 3 years and is now using crutches. Radiologic evaluation at age 13 showed marked osteopenia with thin cortices and varus deformity and bowing of the femora. She suffered 6 more fractures between age 11 and 16 years. Now, at age 26, she has developed a severe thoracic and lumbar scoliosis. Her stature is 152 cm, her sclerae are white and hearing is normal. Her phenotype is compatible with OI type IV.

Proband MJ (fig. 2A) was first seen by us at age 40. She was born at term after an uneventful pregnancy. Reportedly, she suffered her first fracture at 3½ months. She had severe difficulties walking since early childhood, moved with crutches at age 10 and became wheelchair-bound at age 14. She suffered more than 40 fractures, mostly of

the lower extremities. The fractures were treated with casts and always involved long periods of immobilisation. By age 38, her hearing had decreased.

Clinical evaluation at age 40 showed that she was severely dwarfed with a stature below 1.40 m and presented with severe shortening and bowing of her lower extremities. In addition, she had a large head with triangular face, white sclerae, opalescent teeth, joint laxity, severe thoracic hyperkyphosis and lumbar hyperlordosis and flexion contractures of the hips. In contrast, the humeri appeared relatively well modelled and less short. She now suffers from digestive problems due to a huge herniation of the bowel into the thoracic cage. Her phenotype is compatible with OI type III.

Proband KJ (fig. 2B) is the first child of healthy, non-consanguineous parents. The diagnosis of OI was made on ultrasound in the 7th month of pregnancy. Born at term, he presented two fractures and a birth length of 48 cm. He suffered at least 6 more fractures in the first years of life, had delayed motor development and walked independently by age 3. Examination at that time showed a stature below the 3rd percentile, a large turriccephalic skull, enlarged fontanelles, slightly blue sclerae, low-set ears, dentinogenesis imperfecta, short extremities and mild joint hyperlaxity. Radiological examination disclosed multiple wormian bones, several old rib fractures, very short humeri and mild shortening and anterior bowing of the femora and tibiae. Now, at age 4, he is walking independently without physical impairment. His phenotype is OI type IV.

Biochemical Studies

Skin biopsies were obtained from the 5 OI probands (AU, PT, WN, KJ, MJ), and from the parents of patients WN and AU. Fibroblast cultures were established under standard conditions. After

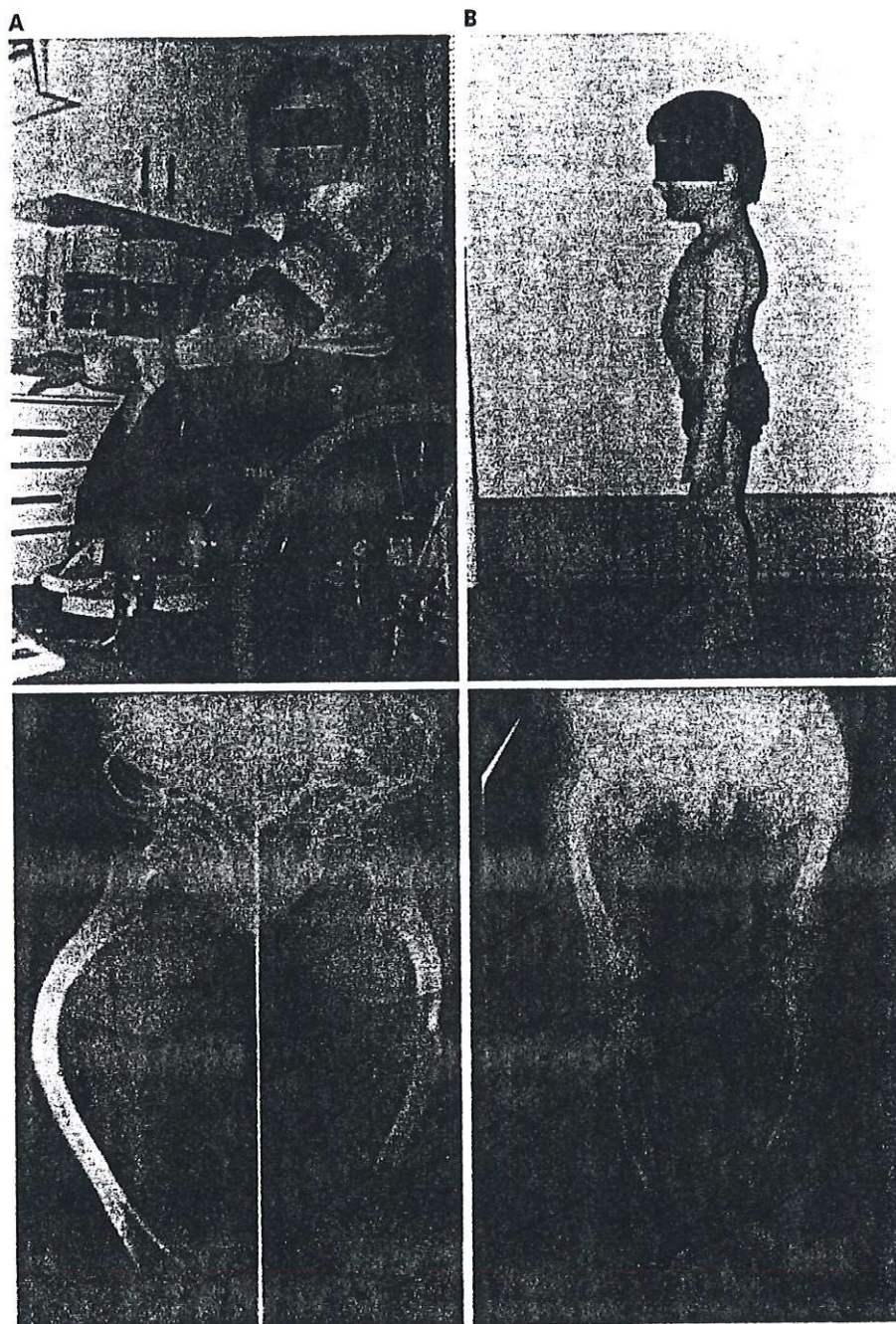


Fig. 2. Clinical and X-rays of patients MJ and KJ with a $\alpha 2(I)$ -G238S substitution. **A** Patient MJ at age 45, showing severe osteopenia and deformation of pelvis and the long bones of the lower extremities. **B** Patient KJ at age 4, already showing distinct bowing of the femora and tibiae.

labelling with ^{14}C -proline, the procollagen and collagen proteins were isolated from medium and cell layer and examined by SDS electrophoresis as described earlier [4]. Thermal stability measurements were performed as described by Bruckner and Prockop [5]. Collagen secreted into the medium was subjected to peptide mapping by in situ cyanogen bromide digestion followed by electrophoresis in the second dimension [6]. Fibroblast cultures labelled in the presence of 0.3 mM $\alpha\alpha'$ -dipyridyl were harvested the same way and evaluated by SDS-PAGE.

Molecular Investigations

Total RNA was isolated from cultured fibroblasts, and cDNA was obtained, using M-MLV reversed transcriptase (Life Technologies) according to the manufacturer's instructions. PCR was performed using primers for the COL1A2 coding region and the amplimers were analyzed by single strand conformation polymorphism analysis (SSCP) as described [7] or by heteroduplex analysis using conformation-sensitive gel electrophoresis (CSGE) as described [8].

Fragments showing an abnormal migration pattern were cloned using pCR-Script cloning kit (Stratagene) and sequenced by the dideoxy chain termination method [9] (numbering starts from the

first Gly residue in the $\alpha 2(I)$ collagen chain as described in the EMBL sequence Z 74616).

The presence of the mutation was investigated by restriction enzyme digestion on cDNA and, where possible, on genomic DNA from the patients and the parents. Genomic DNA was isolated from peripheral blood leukocytes using the Qiagen-Blood miniprep kit (Qiagen Inc. Chatworth, Calif., USA).

Results

Biochemical Collagen Analysis

SDS electrophoresis of pepsin-digested collagen from the medium showed slightly altered collagen profiles for each of the probands, compared to the control samples (fig. 3A). In patients AU, WN, PT and KJ, broadening of the bands representing the $\alpha 1$ - and/or $\alpha 2$ -chains of type I collagen was seen, suggesting that in addition to normal α -chains a population of slowly migrating overmodified α_M -chains was synthesized. However, the degree of overmodification was much more pronounced in AU than in the other OI probands. In patients WN and MJ, the intensity of the $\alpha 2(I)$ band was decreased and in patient WN, a mild delay in electrophoretic mobility of the $\alpha 2(I)$ chains was visible. Analysis of the cell layer collagens (fig. 3B) revealed patterns similar to the medium collagens, except in patient MJ, in whom a mild broadening of the $\alpha 2(I)$ chains was seen, compatible with retention of mutant collagen chains in the cells.

Analysis of the procollagens showed a variable delay in processing of procollagen type I in the 5 probands as compared to controls (data not shown).

SDS-PAGE of collagens extracted from fibroblast cultures labelled in the presence of $\alpha\alpha'$ -dipyridyl showed that the slow-migrating type I collagen α -chains disappeared (data not shown), indicating that enzymatic overmodification was indeed the cause of the delayed mobility. The collagen type I melting profiles showed a slight decrease of 1–2°C in denaturing temperature in all OI probands (table 1).

In situ two-dimensional peptide mapping of the type I collagen chains showed that enzymatic overmodification started in the $\alpha 1(I)$ CB6 peptide for patient AU, in the $\alpha 1(I)$ CB7 peptide for patients PT and WN and in the $\alpha 1(I)$ CB8 peptide for patient KJ, suggesting that either the mutation was localized in one of those $\alpha 1(I)$ CB peptides or to the corresponding region of the $\alpha 2(I)$ chain (data not shown).

Molecular Analyses

Using primers covering the complete COL1A1 and COL1A2 α -helix coding region, RT-PCR was performed

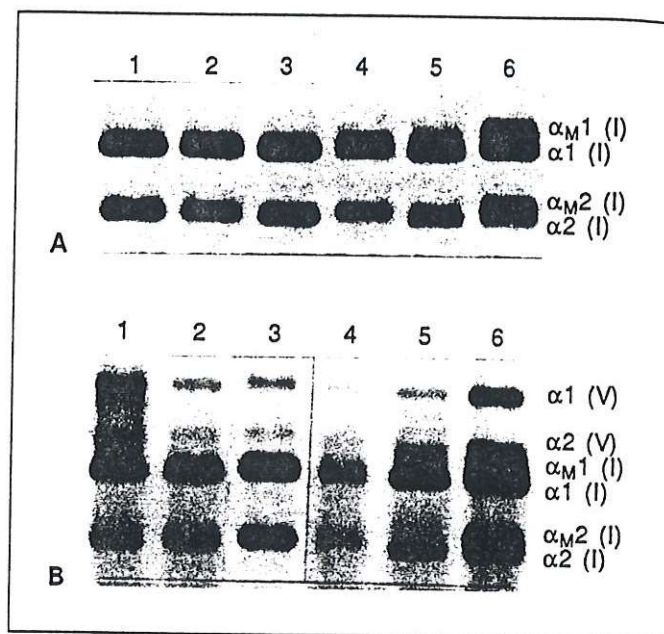


Fig. 3. Analysis by SDS-PAGE of pepsin-derived collagens secreted by cultured fibroblasts. Lane 1: control samples; lane 2: patient MJ; lane 3: patient KJ; lane 4: patient WN; lane 5: patient PT, and lane 6: patient AU. $\alpha 1(I)$ and $\alpha 2(I)$: normal collagen type I α -chains: $\alpha 1(I)_M$ and $\alpha 2(I)_M$: mutant, more slowly migrating collagen type I α -chains. **A** Electrophoresis of the collagen secreted into the medium showed broadening of the bands representing the $\alpha 1(I)$ and $\alpha 2(I)$ chains in all patients, except in patient MJ. The band representing the $\alpha 2(I)$ chains was decreased in intensity in patients MJ (lane 2) and WN (lane 4), and showed delayed migration in patient WN. **B** Cell-layer collagens derived from the 5 OI patients showed varying broadening of the bands representing the collagen type I α -chains. In patient MJ, broadening of the collagen α -chains is seen in contrast to the normal findings in the medium, suggesting retention of the mutant collagen type I chains in the cell layer.

on mRNA derived from the patients' fibroblasts. Migration shifts were detected in the COL1A2 gene by SSCP in the patients MJ, WN and AU and by heteroduplex analysis in patients KJ and PT. Cloning and sequencing of the relevant COL1A2 fragments resulted in the identification of 4 different G to A transitions, which changed the codon for glycine to that for serine (table 1): a G238S in patients MJ and KJ, a G682S in patient WN, a G811S in patient PT and a G859S in patient AU. The presence of the mutation was confirmed by restriction analysis on cDNA in the 5 OI patients, and on genomic DNA isolated from leukocytes in patients MJ, KJ and WN. No mutation was found in the parents of patients MJ, KJ and WN. Genomic DNA from all family members of patient PT were analyzed by restriction digestion, but the $\alpha 2$ -G811S substitution was not present.

Table 1. Position of the serine for glycine substitution in the collagen $\alpha 2(I)$ chain characterized in the different OI probands, the resulting OI phenotype and the decrease in melting temperature of mutant versus normal type I molecules

Patient	Nucleotide substitution	Nucleotide position	Glycine residue	OI phenotype	Decrease in thermal stability, °C
MJ	GGT $\rightarrow$ AGT	1121	238	III	1.5
KJ	GGT $\rightarrow$ AGT	1121	238	IV	2.0
WN	GGT $\rightarrow$ AGT	2453	682	IV	1.5
PT	GGC $\rightarrow$ AGC	2840	811	IV	1.0
AU	GGT $\rightarrow$ AGT	2984	859	IIB/III	1.0

Discussion

Substitutions of serine for glycine in collagen type I are the most common single base substitutions detected in OI. So far, the number of serine substitutions identified in the $\alpha 1(I)$ chain by far exceeds that of serine substitutions in the $\alpha 2(I)$ collagen chain, as shown by the data of the Osteogenesis Imperfecta Mutation Consortium (OIMC) (fig. 4).

The 5 OI probands described here all harbor substitutions of serine for glycine in the $\alpha 2(I)$ collagen chain, resulting from a heterogeneous G $\rightarrow$ A transition at a CpG nucleotide. Pruchno et al. [10] have suggested that CpG dinucleotides within the coding region of the type I collagen genes represent 'hot spots' for mutation. This is supported by the fact that all recurrent mutations described so far in OI involve CpG dinucleotides.

In the 5 patients described here, the OI phenotype most probably results from a new mutation since in all of them the family history is negative. In particular, in patients MJ, KJ and WN, no evidence for mosaicism was found as a possible cause for the differences in phenotypic severity in these patients.

The biochemical profiles are in agreement with the degree of overmodification as expected from the position of the substitution of the serine for glycine along the $\alpha 2(I)$ collagen chain. In 2 patients, MJ and WN, a reduced amount of $\alpha 2(I)$ collagen chains was observed. In the first, MJ, this can be explained by intracellular retention of the mutant $\alpha 2(I)$ collagen chains (fig. 3B). In patient WN, there is no evidence for intracellular accumulation, but rapid degradation of mutant $\alpha 2(I)$ chains may explain the reduction of $\alpha 2(I)$ collagen chains in the medium.

The serine substitutions span a major part of the $\alpha 2(I)$ collagen helix. The substitution closest to the C terminus, G859S, causes the most severe OI phenotype, whereas G811S and G682S produce a moderately severe phenotype and the N-terminal G238S substitution is associated with a moderately (patient KJ) and a severely deforming (patient MJ) OI phenotype. Three of the serine substitu-

tions have been reported earlier in OI patients. The G859S substitution was previously detected in a patient with the severe OI type III phenotype, as seen in patient AU [11]. The G811S substitution causing moderately severe OI in patient PT was previously documented in a patient with lethal OI [Byers et al., pers. commun.]. The G238S substitution (patients KJ and MJ) was reported respectively in patients with OI type III [12], OI type I and OI type I/IV [Byers et al., pers. commun.]. This mutation may represent a 'hot spot', as it has also been detected in several other OI patients [Byers et al., pers. commun.]. Differences in phenotypic severity resulting from the same glycine substitution have been detected not only in unrelated OI patients [13, 14] but also in patients from the same family [15]. An explanation for these observations is not found yet, but variation in genetic background as well as differences in levels of gene expression may be involved. Dyne et al. [16] described deficient expression of decorin in a case of severe/lethal OI presenting a $\alpha 1(I)$ -G415S substitution. The clinical outcome in this particular patient was much more severe compared to 2 other OI patients with the same substitution but with normal decorin expression levels. Sakai et al. [17] discussed the role of as yet unidentified 'modifiers' in the molecular, intra- and extracellular pathways as factors influencing phenotypic variability. In addition to this, differences in therapeutic management have most probably also contributed to some extent to the phenotypic differences between patients KJ and MJ. Indeed, the long periods of immobilisation and lack of an adequate physical rehabilitation programme most certainly worsened the physical condition of patient MJ as compared to patient KJ, who has received early intensive orthopaedic care.

The mutations reported here add to the data suggesting that although a gradient of clinical severity from the carboxyl- to amino-terminal end is generally observed for collagen type I mutations, there are exceptions to this model, in particular for glycine to serine substitutions irrespective of the type of chain involved.

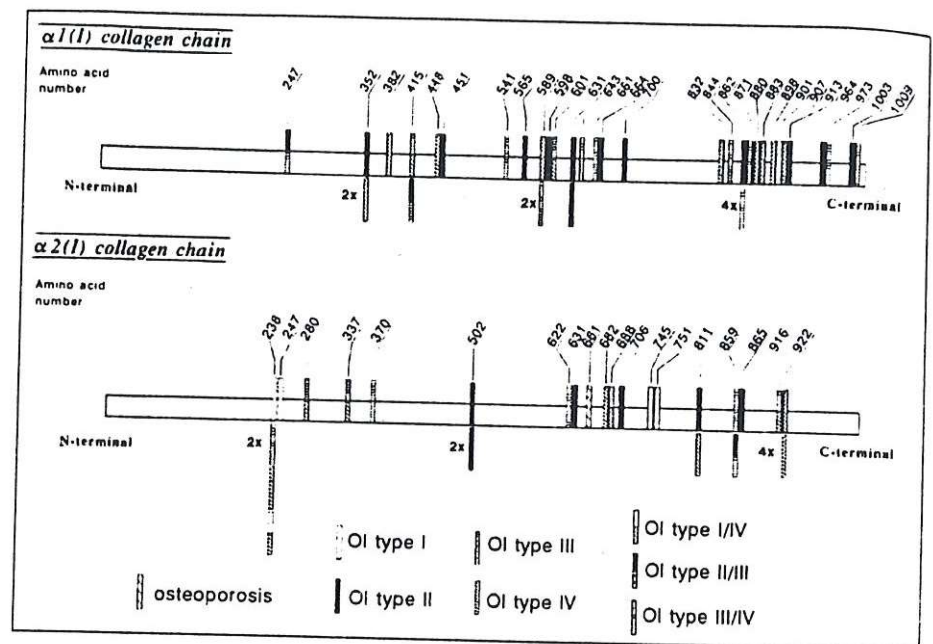


Fig. 4. Diagram summarizing serine for glycine substitutions in the $\alpha 1(I)$ and $\alpha 2(I)$ collagen chain, as reported to the OIMC (September 1996).

$\alpha 1(I)$ collagen chain	$\alpha 2(I)$ collagen chain
G247S Mackay et al., Hum Mol Genet 1993;2:1155-1160	G238S Rose et al., Hum Genet 1995;95:215-218
G352S Bateman et al., Biochem J 1992;288:131-135	G238S Byers, pers. commun.
G352S Marini et al., J Biol Chem 1993;268:2667-2673	G328S Byers, pers. commun.
G352S Mackay et al., Hum Mol Genet 1993;2:1155-1160	G238S reported here
G382S Mackay et al., Hum Mol Genet 1993;2:1155-1160	G247S Zhuang et al., Hum Mut 1996;7:89-99
G415S Bateman et al., Biochem J 1992;288:131-135	G280S Lund, pers. commun.
G415S Mottes et al., Hum Mut 1993;2:196-204	G337S Byers, pers. commun.
G448S Byers, pers. commun.	G370S Zhuang et al., Hum Mut 1996;7:89-99
G451S Byers, pers. commun.	G502S Rose et al., Hum Genet 1994;94:497-503
G541S Mackay et al., Hum Mol Genet 1993;2:1155-1160	G622S Lund, pers. commun.
G565S Bateman et al., Biochem J 1992;288:131-135	G631S Byers, pers. commun.
G589S Gomez-Lira et al., V Intern Conf OI, 1993	G661S Spotila et al., Proc Natl Acad Sci USA 1991;88:5423-5427
G589S Zhuang et al., Hum Mut 1996;7:89-99	G682S reported here
G589S Lund, pers. commun.	G688S Raghunath et al., Eur J Pediatr 1995;154:123-129
G598S Westerhausen et al., J Biol Chem 1990;265:13995-14001	G706S Wang et al., J Biol Chem 1993;268:25162-25167
G601S Lund, pers. commun.	G745S Zhuang et al., Hum Mut 1996;7:89-99
G631S Westerhausen et al., J Biol Chem 1990;265:13995-14001	G751S De Paepe et al., Hum Genet 1997;99:478-483
G631S Byers, pers. commun.	G811S Byers, pers. commun.
G643S Lund et al., Hum Mut 1997;9:378-382	G811S reported here
G661S Nuytinck et al., Hum Genet 1996;97:324-329	G859S Rose et al., Hum Mut 1990;3:391-394
G664S Byers, pers. commun.	G859S reported here
G700S Byers, pers. commun.	G865S Lamande et al., J Biol Chem 1989;264:15809-15812
G832S Marini et al., J Biol Chem 1989;264:11893-11900	G916S Byers, pers. commun.
G844S Pack et al., J Biol Chem 1989;264:19694-19699	G922S Marini et al., J Biol Chem 1993;268:2667-2673
G862S Virdi et al., Hum Genet 1994;93:287-290	G922S Sztrolovics et al., Hum Mol Genet 1993;2:1319-1321
G862S Zhuang et al., Hum Mut 1996;7:89-99	G922S D'Amato et al., V Intern Conf OI, 1993
G862S Nuytinck and De Paepe, unpubl.	G922S Byers, pers. commun.
G862S Lund et al., Hum Mut 1997;9:378-382	G922S Byers, pers. commun.
G862S Namikawa et al., Hum Genet 1995;95:666-670	
G871S Lund et al., Hum Mut 1997;9:378-382	
G880S Lund et al., Hum Mut 1997;9:378-382	
G883S Lightfoot et al., Int J Exp Pathol 1993;74:A11-A12	
G898S Lund et al., Hum Mut 1997;9:378-382	
G901S Mottes et al., Hum Genet 1992;89:480-484	
G907S Nuytinck and De Paepe, unpubl.	
G913S Cohn et al., Matrix 1990;10:236 (abstract)	
G964S Wallis et al., Am J Hum Genet 1989;45:A228 (abstract)	
G973S Gomez-Lira et al., V Intern Conf OI, 1993	
G1003S Pruchno et al., Hum Genet 1991;87:33-40	
G1009S Cohn et al., Matrix 1990;10:236 (abstract)	

Three substitutions of serine for glycine have been identified as well in the $\alpha 1(I)$ as in the $\alpha 2(I)$ collagen chain, i.e. the G247S, G631S and G661S substitutions [7, 18–21; Byers et al., pers. commun.]. The $\alpha 1(I)$ G247S and G661S substitutions cause distinctly more severe phenotypes than their homologues in the $\alpha 2$ -chain, which is explained by the fact that the type I collagen molecule contains two $\alpha 1$ -chains and one $\alpha 2$ -chain. However, this is not the case for the G631S substitution which causes a lethal phenotype in all instances [19; Byers et al., pers. commun.]. This illustrates that besides the chain effect, other domain-related factors determine the phenotypic outcome of a glycine substitution in type I collagen. There are 'lethal' regions which are crucial for the structural and functional integrity of the molecule and 'non-lethal' regions, in which sequence variations have a less dramatic effect on the molecular stability function. Apparently, these regions do not necessarily correspond for both collagen type I α -chains, suggesting that these regions are chain specific, rather than molecule specific. A possible explanation

for this phenomenon is the specific chain bonding of yet unidentified molecules which play a role in the intra- and extracellular pathways and hence contribute to correct fibril formation and function.

In conclusion, the phenotypic outcome of collagen type I mutations, as illustrated here for glycine to serine substitutions, is influenced by many different factors. Besides the position and nature of the mutation, other effects such as epigenetic factors and environmental influences determine the phenotypic outcome and make it difficult to predict the OI phenotype from the genotype.

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