Human Molecular Genetics, 1996, Vol. 5, No. 9 **1339–1343**

A family with Stickler syndrome type 2 has a mutation in the *COL11A1* gene resulting in the substitution of glycine 97 by valine in α1(XI) collagen

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Received May 8, 1996; Revised and Accepted June 25, 1996

Stickler syndrome (hereditary arthro-ophthalmopathy) is the commonest inherited cause of retinal detachment and one of the commonest autosomal dominant connective tissue dysplasias. There is clinical and locus heterogeneity with about two thirds of families linked to the gene encoding type II procollagen (*COL2A1*). Families with Sticklers syndrome type 1 have a characteristic congenital vitreous anomaly and are linked without recombination to markers at the *COL2A1* locus. In contrast families with the type 2 variety have a different vitreo-retinal phenotype and are not linked to the *COL2A1* gene. Type XI collagen is a quantitatively minor fibrillar collagen related to type V collagen and associated with the more abundant type II collagen fibrils. A mutation in *COL11A2*, the gene for α2 (XI) procollagen, has recently been found in a family described as having Stickler syndrome, although there was no ocular involvement. Here we show for the first time that a family with the full Type 2 Stickler syndrome including vitreous and retinal abnormalities is linked to the *COL11A1* gene and characterise the mutation as a Glycine to Valine substitution at position 97 of the triple helical domain caused by a single base G→T mutation. These results are the first to provide confirmation that type XI collagen is an important structural component of human vitreous. They also support previous work suggesting that mutations in the genes encoding collagen XI can give rise to some manifestations of Stickler syndrome, but of these, only mutations in *COL11A1* will give the full syndrome including the vitreo-retinal features.

INTRODUCTION

Stickler syndrome (hereditary arthro-ophthalmopathy) is an autosomal dominant condition characterised by ocular, articular, facial, auditory and oral features. It is the commonest autosomal dominant connective tissue dysplasias (1) and the commonest cause of inherited retinal detachment (2). There is clinical and locus heterogeneity with about two thirds of families showing linkage to the gene encoding Type II procollagen (*COL2A1*). We have recently shown that Stickler syndrome can be sub classified on the basis of vitreo-retinal phenotype: Type 1 families with a characteristic congenital vitreous anomaly show linkage without recombination to markers at the *COL2A1* locus; Type 2 families with different congenital vitreo-retinal phenotypes are not linked to *COL2A1* (3).

Type XI collagen is a quantitatively minor fibrillar collagen related to type V collagen and associated with the more abundant type II collagen fibrils (4–6). A mutation in *COL11A2*, the gene for α2 (XI) procollagen, has recently been found in a family described as having Stickler syndrome (7), although there was no ocular involvement. In cartilage the type XI collagen molecule is a heterotrimer composed of α1, α2 and α3 (XI) chains. However in mammalian vitreous the α2 (XI) chain is replaced by α2 (V) collagen (8). In addition it is now clear that the α3 (XI) collagen chain is a splice variant of the *COL2A1* gene (4,9,10). Therefore, the *COL11A1* and *COL5A2* genes were strong candidates for Type 2 Stickler syndrome.

We now report the results of a study in a large Type 2 pedigree in which linkage to *COL2A1* had been excluded. Slit lamp biomicroscopy of affected members of the pedigree revealed congenitally abnormal vitreous architecture suggesting that likely candidates would be the genes encoding other collagens that associate with, and structurally stabilise, type II collagen.

RESULTS

The four generation Stickler syndrome Type 2 family studied consisted of seven affected and nine normal individuals. All affected individuals had the characteristic ocular, auditory and oro-facial features of Stickler syndrome (Fig. 1). Abnormal vitreous architecture is the hallmark of the syndrome and was considered a prerequisite for the diagnosis. It was present in all affected individuals; in each case the myopia was congenital, non-progressive and of high degree.

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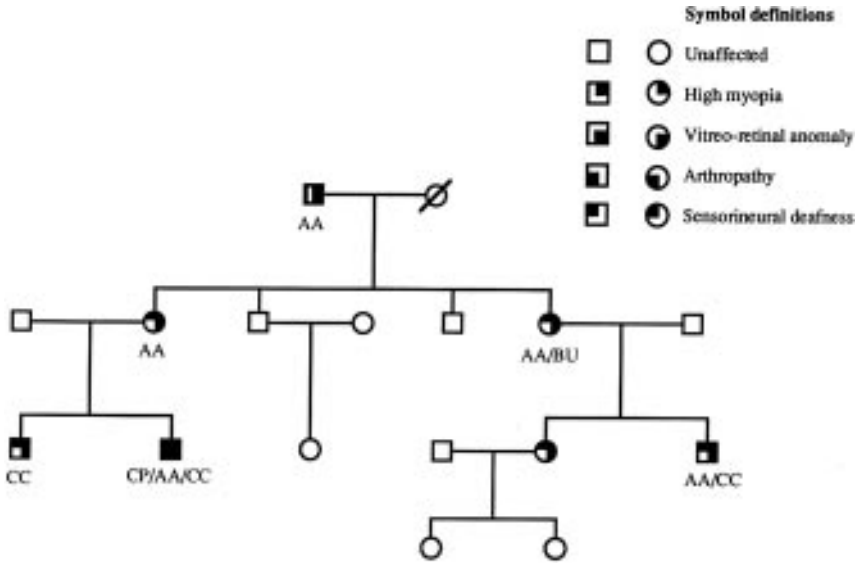

Figure 1. Type 2 Stickler syndrome pedigree. Symbols show ocular, auditory, articular, and oral features of affected individuals.

Table 1. Two point lod scores between Type 2 Stickler syndrome and polymorphic markers

			Recombination fraction						
Locus	Location	Marker	0.0	0.001	0.05	0.1	0.2	0.3	0.4
COL2A1	12q12-q13.2	3′VNTR	–∞	–7.2	–2.7	–1.3	–0.6	–0.2	–0.1
CRTL1	5q13-q14	[GT] repeat	–∞	–10.2	–3.4	–2.3	–1.2	–0.6	–0.2
COL3A1	2q14-q32	IVS 25	–∞	–7.6	–2.6	–1.8	–1.1	–0.7	–0.4
COL9A1	6q12-q14	[CA] repeat	–∞	–13.2	–4.7	–3.2	–1.8	–1.0	–0.4
COL11A2	6p21.3	*D6S105*	–∞	–4.8	–1.4	–0.9	–0.4	–0.1	0.0
		D6S276	–∞	–6.6	–1.6	–0.8	–0.2	–.06	–0.1
COL11A1	1p21	*D1S206*	1.2	1.2	1.0	0.9	0.5	0.2	0.0
		D1S223	2.7	2.7	2.5	2.2	1.7	1.1	0.6

Genomic DNA was extracted from peripheral blood of all 16 family members. In addition skin biopsies from II-6 and III-2 (Fig. 1) were used to obtain dermal fibroblast cultures. Polymorphic markers within or close to the *COL2A1*, *COL5A2*, *COL11A1* and *COL11A2* genes were analysed as well as the gene (*CRTL1*) for the proteoglycan link protein which plays an integral role in the stabilisation of cartilage extracellular matrix and has recently been linked to Wagner's disease and Erosive Vitreoretinopathy (11).

Analysis of the *COL2A1* VNTR polymorphism (12) showed at least three recombinants with the disease which excluded linkage to *COL2A1* up to a recombination fraction of 0.05 (Table 1). This region of exclusion would extend to the flanking marker *D12S18* distally and well beyond the *COL2A1* gene which encompasses only 30 kb (13). Linkage analysis of a polymorphic $[GT]_n$ repeat in the 5′ promoter region of *CRTL1* (14) showed several recombinations and was also excluded (Table 1).

COL5A2 maps to 2q14-q32, the same region of chromosome 2 as *COL3A1* (15). A highly polymorphic sequence from intron 25 of *COL3A1* (16) was amplified by PCR and used as a marker for *COL5A2*. Linkage was excluded by the demonstration of at least two recombinations between the disease and *COL5A2* locus. Using the polymorphic markers *D6S105* and *D6S276* the *COL11A2* locus at 6p21 was also excluded by recombination between the disease and marker loci (Table 1).

Linkage to *COL11A1* was tested with two $[CA]_n$ repeat polymorphisms *D1S223* and *D1S206*. These markers are 2 cM from the *COL11A1* gene (Warman, M.L., Tiller, G., pers. comm.) at 1p21 (17). With *D1S223*, all meioses were informative and no recombinants were detected, giving a maximum lod score of 2.7. Analysis with *D1S206* was only partially informative giving a maximum lod score of 1.2 at zero recombination. These data strongly suggested the disease locus to be *COL11A1*.

Mutational analysis of *COL11A1* was performed on RT–PCR products using RNA extracted from cultured dermal fibroblasts. In total 14 overlapping cDNA products, covering the entire open reading frame, were analysed. Single stranded conformational analysis of cDNA product 5 indicated sequence variation in affected individuals which was absent in normal controls (data not shown). When this PCR product was directly sequenced a heterozygous single base change was observed (Fig. 2) which substituted glycine 97 by valine and disrupted the normal

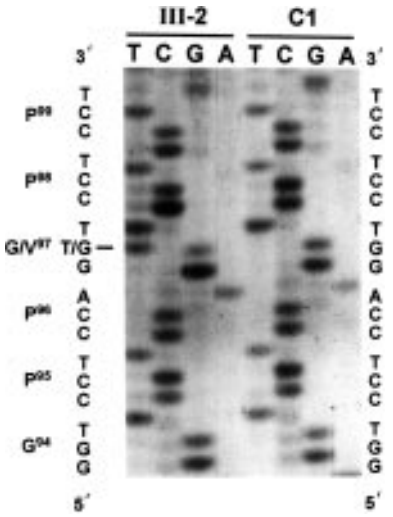

Figure 2. Sequence analysis. Sequence obtained by direct sequencing of RT–PCR product 5 from III-2 and a normal control (C1). The heterozygous T/G base in the patient is indicated and the alteration to the amino acid sequence shown.

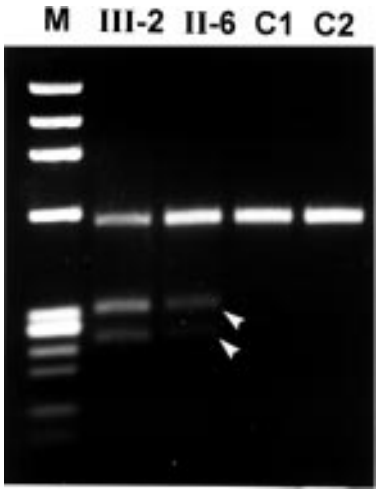

Figure 3. cDNA analysis. The RT–PCR product 5 from II-6, III-2 and two normal controls (C1, C2) was incubated with restriction enzyme *Bsr*I and then analysed by agarose gel electrophoresis, along with known standards (M). The two fragments produced by the new *Bsr*I site are indicated by arrow heads.

Gly-X-Y collagen sequence (the normal convention of numbering the first glycine of the triple helix as 1 has been used). This base change creates a *Bsr*I restriction enzyme site and incubation of the cDNA with this enzyme confirmed the presence of the mutation in the RT–PCR products (Fig. 3). The gene structure of *COL11A1* has not yet been determined, however the corresponding region in *COL11A2* would lie within exon 19 (18). Genomic DNA from each family member was amplified using primers 5′ and 3′ of the base change, in regions likely to lie within exons 18 and 20. A product of 1.4 kb was obtained. The enzyme *Bsr*I cut the DNA normal DNA into three fragments of approximately 525, 475 and 400 bp. In affected individuals an extra *Bsr*I site was detected which cut the 400 bp band approximately in half, resulting in two fragments of around 200 bp, which appear as a single band. The seven affected individuals all possessed the extra *Bsr*I site and must consequently carry the glycine to valine substitution (Fig. 4). Analysis of 100 chromosomes from 50 unrelated controls produced only the pattern of bands seen in the normal family members (data not shown).

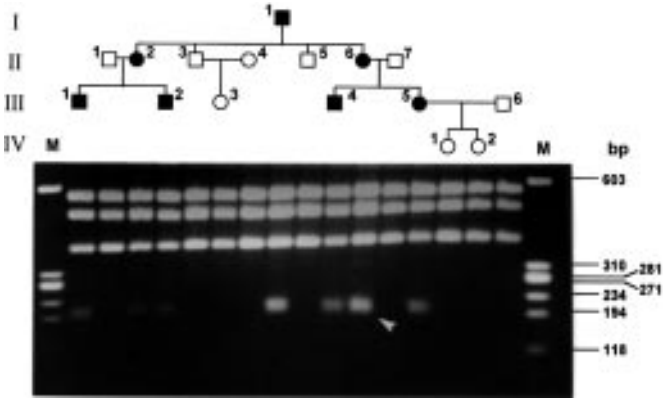

Figure 4. Genomic analysis. Amplified genomic DNA containing the proposed exon 19 was amplified from each family member and incubated with *Bsr*I. The products were analysed by agarose gel electrophoresis, along with standard markers (M) as indicated. The extra band present in digested DNA from affected individuals is indicated by the arrow head.

DISCUSSION

The evidence that this base change in *COL11A1* is the causative mutation in this pedigree is four fold. Firstly, Sticklers syndrome Type 1 is consistently caused by mutations of *COL2A1* (19,20). Type XI collagen is known to associate with type II collagen fibers and it is now known that the α3(XI) collagen chain is a product of the *COL2A1* gene. This implies a functional relationship between collagens II and XI. Secondly, mutations of α2(XI) collagen, which is not expressed in the vitreous, cause a Stickler-like phenotype but without any ocular involvement (21). Thirdly, linkage analysis with markers close to the *COL11A1* gene were fully informative and produced a maximum lod score of 2.7, which strongly implied a causative association with the *COL11A1* gene. Fourthly, there are numerous precedents in other collagen genes which show that substitutions of triple helical glycines cause inherited disorders of the extracellular matrix (19,20,22,23). Furthermore such change does not occur in the normal general population.

The mutation will have a dominant negative effect, since it will disrupt the function of normal gene products with which the abnormal α1(XI) associates, namely α2(XI), α3(XI) and α2(V) collagens. Thus half of the α1(XI) containing heterotrimers will contain the mutant protein. In contrast all type 1 Stickler mutations of α1(II)/α3(XI) have so far been caused by premature termination codons in *COL2A1*. This causes haploinsufficiency with half normal amounts of wild type α1(II) and α3(XI) collagens, but no protein capable of collagen trimer formation. Other dominant negative mutations of *COL2A1* have caused more severe disorders such as Kniest dysplasia and achondrogenesis (19,20) because they affect not only the α3(XI) chain but also the more abundant homotrimer $\alpha1(II)_3$ collagen resulting in only 1/8 production of the normal molecule. These permutations may well account for the characteristic and distinct vitreous phenotypes seen in Type 1 and Type 2 Stickler patients. The results from this pedigree provide strong support for previous work (21) suggesting that mutations in the

genes encoding collagen XI can give rise to some manifestations of Type 2 Stickler syndrome. However, on the basis of our results and studies on bovine vitreous (8), it is likely that of these, only mutations in *COL11A1* will give the full syndrome including the vitreo-retinal features. The mutation characterised here is the first described in the *COL11A1* gene and provides a valuable human comparison with the transgenic mice model which express only normal α1(XI) collagen and appear to have a more severe phenotype (24).

MATERIALS AND METHODS

Linkage analysis

All pedigree members underwent full clinical and ophthalmologic examination by two of the authors (MPS, JDS). Informed written consent was received in all cases and prior ethical approval for the study was obtained. The criteria for diagnosis of Type 2 Stickler syndrome were as follows: (i) architecturally abnormal vitreous gel but absence of Type 1 congenital vitreous anomaly in all affected subjects (2); and in addition, any three of the following features: (ii) myopia, stable or progressive, onset at any age; (iii) rhegmatogenous retinal detachment or paravascular pigmented lattice degeneration; (iv) joint laxity with abnormal Beighton score (25) with or without radiological evidence of joint degeneration; (v) audiometric confirmation of sensorineural hearing defect; (vi) high arched or cleft palate.

Leukocyte DNA was extracted from 20–30 ml of peripheral blood according to standard procedures. Analysis of the marker loci was carried out by PCR amplification of genomic DNA using the reported primer sequences in 25 µl reaction volumes. Each reaction contained a forward primer which had been end labeled with γ^{32}P ATP and T4 polynucleotide kinase. Alleles were separated by electrophoresis in 4–6% denaturing polyacrylamide gels and visualised by autoradiography. Lod scores were calculated using the LIPED computer program (26). Autosomal dominant inheritance was assumed with complete penetrance.

cDNA amplification

Total cytoplasmic RNA was isolated from cultured dermal fibroblasts and used to reverse transcribe cDNA as previously described (27). Using the cDNA sequences described by Bernard *et al.* and Yoshika and Ramirez (accession No.J04177) (28,29) 14 cDNAs were amplified. These covered the entire open reading frame as follows, product [1] bases 142–680; [2] 536–1070; [3] 931–1489; [4] 1349–1893; [5]1784–2348; [6] 2196–2740; [7] 2630–3128; [8] 3007–3528; [9] 3418–3924; [10] 3793–4316; [11] 4168–4721; [12] 4604–5114; [13] 4982–5439; [14] 5315–5816. Each product was approximately 500 bp in length and overlapped its 5′ and 3′ neighbours by around 100 bp. Each set of primers corresponded to the first 24 sense and last 24 antisense nucleotides of the 14 products. These were first reverse transcribed using the antisense primer from each product's 3′ neighbour. An initial round of amplification used the same antisense primer and the 5′ sense primer of the desired final product. The resulting cDNA (around 900 bp) was purified using a Quiquick™ spin column (Quiagen) and eluted in 50 µl of 10 mM Tris–HCl pH 8.0, 0.1 mM EDTA. One µl of this was then used to amplify the final product (around 500 bp) using the nested 3′ antisense primer contained within the initial amplification product but the same 5′ primer. The antisense primer used for reverse transcription and primary amplification of product 14 covered bases 6010–6033. All amplification reactions were performed using an Ampliwax™ PCR Gem (Perkin Elmer) to utilise the hot start technique. A final reaction volume of 100 µl contained 20 mM Tris–HCl pH 8.4, 50 mM KCl, 2.5 mM $MgCl_2$, 200 µM of each dNTP, 25 pmol of each primer and 2.5 U *Taq* DNA polymerase. After initial denaturation of 5 min at 95°C, 35 cycles of 95°C 1.5 min, 65°C 1.5 min and 72°C 3 min were used to obtain the cDNA products

Single stranded conformational analysis

Each cDNA product was incubated with restriction enzymes which cut the cDNA at 1–3 sites. For most products two different restriction enzymes were used to generate at least one fragment of a size (around 200 bp or less) suitable for SSCP analysis. After digestion 10 µl of loading buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) was added to the 20 µl reactions, and heat denatured at 97–100°C for 5 min. Electrophoresis was performed at 4°C in a 0.75 mM thick gel which consisted of 0.5 × MDE (Flowgen) 0.6 × TBE buffer. Bands were visualised by silver staining.

Sequencing

For sequencing, a cDNA amplification product was purified on a Quiquick™ spin column and eluted in water. It was then directly cycle sequenced using the Exo(–) Pfu cyclist™ DNA sequencing kit (Stratagene) as recommended by the manufacturers. The products were analysed in a 6% denaturing polyacrylamide gel and autoradiographed.

Restriction enzyme analysis

Genomic DNA from all 16 family members and 50 normal unrelated controls were amplified essentially as described above using the primers X18S (5′gggtttgatggacttccgggtctg) and X20AS (5′tggaagacctcttggtccaatttc) The 1.4 kb products were incubated with the restriction enzyme *Bsr*I at 65°C, as were the product 5 cDNAs from two affected and two normal individuals. These were then analysed by electrophoresis in a 2% agarose gel, stained with ethidium bromide and visualised under UV light.

ACKNOWLEDGEMENTS

The authors are grateful to the many consultants who have referred patients to the vitreo-retinal service at Addenbrooke's Hospital. MPS is in receipt of an Oxford Ophthalmological Congress Research Scholarship and a grant from the Addenbrooke's NHS Trust Endowment Fund. RW is in receipt of a research grant from the Iris Fund for the Prevention of Blindness. The authors are grateful to Dr D.E. Barton for valuable help with grant submission. We thank Dr George Tiller from Vanderbilt University Medical Center, Nashville, TN and Dr Matt Warman from the Department of Clinical Genetics at Case Western Reserve University School of Medicine, Cleveland, Ohio for helpful discussions and primer details for *COL11A1* and *COL11A2*. We thank Dr Alan Nicholls from MRC Connective Tissue Genetics Group, Cambridge for *COL3A1*/*COL5A2* markers.

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