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Stickler syndrome and the vitreous phenotype: Mutations in *COL2A1* and *COL11A1*.

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ABSTRACT: Stickler syndrome is a dominantly inherited disorder affecting the fibrillar type II/XI collagen molecules expressed in vitreous and cartilage. Mutations have been found in *COL2A1*, *COL11A1* and *COL11A2*. It has a highly variable phenotype that can include midline clefting, hearing loss, premature osteoarthritis, congenital high myopia and blindness through retinal detachment. Although the systemic phenotype is highly variable, the vitreous phenotype has been used successfully to differentiate between patients with mutations in these different genes. Mutations in *COL2A1* usually result in a congenital membranous vitreous anomaly. In contrast mutations in *COL11A1* result in a different vitreous phenotype where the lamellae have an irregular and beaded appearance. However, it is now apparent that a new sub-group of *COL2A1* mutations is emerging that result in a different phenotype with a hypoplastic vitreous that fills the posterior chamber of the eye, and is either optically empty or has sparse irregular lamellae. Here we characterise a further 89 families with Stickler syndrome or a type II collagenopathy, and correlate the mutations with the vitreous phenotype. We have identified 57 novel mutations including missense changes in both *COL2A1* and *COL11A1* and have also detected two cases of complete *COL2A1* gene deletions using MLPA. ©2010 Wiley-Liss, Inc.

KEY WORDS: Stickler syndrome, Vitreous, COL2A1, COL11A1

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INTRODUCTION

Stickler syndrome (MIM 108300, 604841, 184840) is a connective tissue disorder usually caused by autosomal dominant mutations in the genes for the fibrillar collagens that are expressed in cartilage and vitreous (Snead and Yates 1999). Patients can present with a multi-system, variable phenotype, that may include premature osteoarthritis, congenital high myopia, hearing loss, as well as craniofacial abnormalities that can include midline clefting (bifid uvula or cleft palate) retrognathia, and malar or midfacial hypoplasia. There is also a high incidence of retinal detachment that can lead to blindness. Mutations have been found in the genes for type II and type XI collagens (*COL2A1* MIM 120140, *COL11A1* MIM 120280, *COL11A2* MIM 120290) that form heterotypic (composite) collagen fibrils (Ahmad et al 1991, Vikkula et al 1995, Richards et al 1996). These have been referred to as type 1, type 2 and type 3 Stickler syndrome respectively. The syndrome has also been subdivided based on the vitreous phenotype resulting from mutations in the various loci. Mutations in COL2A1 most often result in a congenital membranous vitreous anomaly (Snead and Yates 1999) and this phenotype can also be seen in other *COL2A1* disorders (Meredith et al 2007) such as SEDC (MIM 183900), SEMD Strudwick type (MIM 184250) and Kniest dysplasia (MIM 156500). In contrast mutations in *COL11A1* result in a different vitreous phenotype where the gel, that fills the posterior chamber, displays beaded bundles of irregular diameters, probably reflecting the role of type XI collagen in regulating collagen fibrillogenesis (Blaschke 2000, Richards et al 2000a). The other type XI collagen gene *COL11A2* is not expressed in the eye and mutations in this gene result in a non-ocular form of Stickler syndrome (Brunner et al 1994).

Differential diagnosis based upon systemic features is difficult due to variability in the phenotypic expression. Some mutations in *COL11A1* have been classified as Marshall syndrome, but as demonstrated by Annunen et al (1999) the short nose, anteverted nares, midfacial hypoplasia and flat nasal bridge that are common in cases of Marshall syndrome with *COL11A1* mutations, are also often present in young individuals with mutations in *COL2A1*. Making differential diagnosis based on facial phenotypes difficult (Majava et al 2007). In addition, predominantly ocular or non-systemic forms of Stickler syndrome (MIM 609508) exist due to specific types of mutations in *COL2A1*, as well as the non-systemic dominantly inherited rhegmatogenous retinal detachment phenotype (Richards et al 2000b, 2005, 2006), so distinguishing the different gene loci based on the systemic features alone is also unreliable.

As well as the two common vitreous phenotypes, a third has now been identified where the vitreous has less dense, congenitally hypoplastic architecture, compared to the regular compact lamellae that can be seen coursing through the vitreous in young healthy individuals. These have been associated with atypical mutations / phenotypes in *COL2A1*, (Richards et al 2000a, 2002) and occasionally as sporadic instances within families where other affected individuals present with the membranous phenotype (Richards et al 2006). Here we report a family with Stickler syndrome and an unusual hypoplastic vitreous phenotype. We propose a refinement to our strategy to improve mutation identification in this disorder that remains frequently unrecognized and undiagnosed by medical practitioners.

MATERIALS AND METHODS

Clinical assessment

Pedigrees were identified from the Stickler syndrome clinic at Addenbrooke's hospital that receives referrals from throughout the UK. Studies were performed with approval of the local ethics committee (LREC 92/019 and 02/172). A general ophthalmic history was recorded with particular attention to the age of onset, degree and progression of myopia, cataract and vitreoretinal disease. A full ophthalmic examination was carried including slit-lamp biomicroscopy and indirect ophthalmoscopy with scleral depression. A two stage strategy for mutation screening depending upon the vitreous phenotype was followed (Fig. 1). In addition, some families with the beaded vitreous phenotype were also analysed by linkage analysis. The families documented here were screened for mutations over a period of approximately 3.5 years.

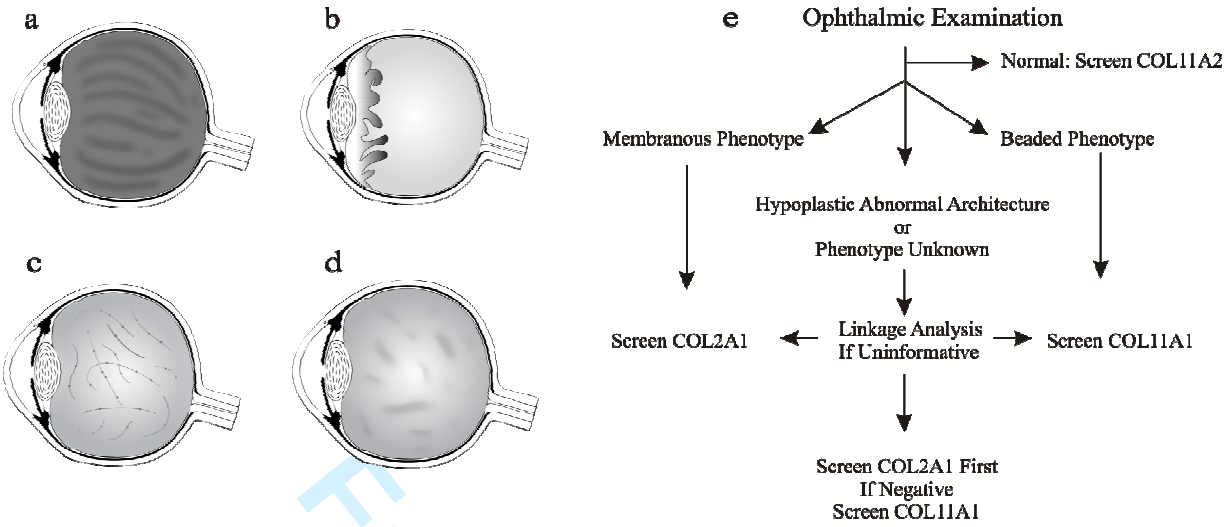

Figure 1. Vitreous phenotypes associated with Stickler syndrome. a) A normal healthy gel appears as a dense homogeneous matrix with lamellae running in the same direction throughout. b) The membranous phenotype consists of a vestigial gel in the retrolental space bounded by a convoluted membrane. c) The beaded phenotype has irregular and thickened lamellae with a beaded appearance. d) The hypoplastic phenotype has a less dense gel matrix which maybe optically empty or with irregular lamellae architecture and, as with the membranous and beaded phenotypes, is a congenital abnormality. e) Flow diagram for gene selection and mutation analysis in Stickler syndrome based on vitreous phenotype.

Linkage analysis

Amplification of variable microsatellite markers close to the genes for *COL2A1* (D12S85, D12S1661, D12S361) and *COL11A1* (D1S2699, D1S2896, D1S2626) were used for linkage analysis to exclude either of these genes as the mutant locus in certain families. Amplified products were analysed using a CEQ 8000 analyser (www.beckman.com) and gene marker software (www.softgenetics.com).

Mutation screening

COL2A1

Screening of *COL2A1* was performed either as previously described (Richards et al 2006) using large PCR products and internal primers for sequencing, or as a high throughput system whereby all 54 exons and the promoter region were amplified simultaneously, as 45 PCR products using a variable buffer system (Masteramp, www.epicentre.com) in a single microtitre plate under the same cycling conditions. All products had an M13 sequence tag incorporated onto the 5' end of one of the primers for use in subsequent sequencing. Reactions were subject to denaturation at 95°C 3min, followed by 33 cycles consisting of 95°C 30sec, 60°C 1min, 72°C 1min, and a final elongation period of 72°C 5min. Following amplification excess primers and dNTPs were removed using Ampure™ (www.beckman.com) which as a final step eluted the DNA in 40 µl water. Aliquots were transferred to another microtitre plate and used in cycle sequencing with BigDye version 1.1 sequence reaction mix (www.appliedbiosystems.com) and the M13 sequence, incorporated onto one end of each product, as a primer. Following sequencing cycles, products were purified with Cleanseq™ (www.beckman.com) eluted in 0.05M EDTA and analysed in a 3730 sequence analyser (www.appliedbiosystems.com). All mutations were confirmed in a second amplified product.

MLPA

Gene dosage was determined by multiplex ligation dependant probe amplification (MLPA) using the *COL2A1* probe set P214 and reagents from MRC-Holland (www.mlpa.com). The probes covered regions in or close to exons 1,3,6,8,10,16,18,24,27,29,31,35,38,43,46,49,51 and 54. Amplified products were analysed on a CEQ 8000 machine (www.beckman.com) and Genemarker software (www.softgenetics.com)

COL11A1

COL11A1 was either analysed as previously described, using mRNA from cultured skin fibroblasts followed by RT-PCR and sequencing of cDNA (Martin et al 1999). Or alternatively Genomic DNA was analysed as a high throughput screen as described above for *COL2A1*, except that the 68 exons, including the alternatively spliced exons 6A and 6B (Annunen et al 1999) were amplified as 61 PCR products. After denaturation at 95°C 2min, amplification was achieved through 35 cycles each consisting of 95°C 30sec, 55°C 30sec 72°C 1min, followed by a final elongation period of 72°C 5min. All mutations were confirmed in a second amplified product.

Genotyping

Over 180 ethnically matched control chromosomes were used to examine the *COL2A1* c.2862C>T, and *COL11A1* c.2755+5G>A mutations. The *COL11A1* intron 35+5G>A mutation was genotyped by amplification of genomic DNA and restriction enzyme analysis using Nde I that cut the mutant but not the normal sequence. The *COL2A1* mutation was analysed using Taqman probes labelled with either 6FAM or VIC using a chromo 4 machine and opticon monitor software (www.bio-rad.com).

Splicing reporter analysis

Splicing reporter analysis of a silent mutation in exon 42 c.2862C>T p.G954 was performed essentially as previously described (Richards et al 2005). Exons 40-44 were amplified, the normal and mutant alleles were cloned into the expression vector pcDNA3.1/Myc/His/A (www.invitrogen.com). Mutant and normal clones were transfected into cultured ARPE-19 cells as previously described (Richards et al 2005) after 24hr cells were harvested and RNA prepared. From this RNA, cDNA was synthesised using a vector specific primer, and amplified with second upstream vector specific primer and one corresponding to exon 44 sequence. Products were sequenced and also analysed by electrophoresis in a 5% polyacrylamide gel. Re-amplification of these products was also performed using nested primers in exons 40 and 43.

RESULTS

The families with mutations documented here were screened over a period of approximately 3.5 years between 2006 and 2009. During that time we failed to detect mutations in 3 cases of patients with the membranous anomaly and 5 cases with the beaded vitreous phenotype. Individuals with normal vitreous architecture were not screened for either *COL2A1* or *COL11A1* mutations. Combined with previous results, the efficiency of mutation detection after vitreoretinal assessment was 96.5% for the membranous phenotype / *COL2A1* and 80% for the beaded phenotype / *COL11A1*, in over 150 cases of Stickler syndrome.

COL2A1 / Membranous vitreous phenotype

The majority, 71/86, of the patients/families with Stickler syndrome displayed the membranous vitreous anomaly associated with mutations in *COL2A1*. In these cases *COL2A1* was amplified, sequenced and analysed for mutations and these are summarised in Table. 1. Most (46/71) resulted in premature termination codons either by point mutations or frameshifts. As has previously been documented a subset (17/71) altered consensus splice sites, and a second example of a cryptic acceptor splice site within intron 23 was also found (Richards et al 2006). In addition the membranous vitreous anomaly was seen in three patients with SEDC who also have mutations in the *COL2A1* gene and are similarly at risk of retinal detachment (Meredith et al 2007). These patients, with severe chondrodysplasia, usually have dominant negative mutations (typically substitutions of glycine) in the *COL2A1* gene, and this was confirmed in a number of new cases (MS266, MS304 and MS306 Table. 1). However, similar novel missense mutations resulting in substitution of glycines p.G240D (MS137) and p.G270R (MS121) were also detected in cases of Stickler syndrome (we have previously briefly reported the p.G270R mutation, Richards et al 2008).

We have also characterised a second silent mutation in *COL2A1* that resulted in missplicing of the mRNA. Like the first case, which resided in exon 30 (Richards et al 2007), this mutation altered the last base of a glycine codon from C>T and created a donor splice site within exon 42. The same mutation was seen in two other individuals with Stickler syndrome, but not in over 180 normal control chromosomes and was not present in the SNP database (http://www.ncbi.nlm.nih.gov/). Splicing reporter analysis demonstrated utilisation of the GT dinucleotide, within the exon, as a donor splice site that resulted in a frameshift (Fig. 2). We have also identified an additional mutation

of the initiation codon, unlike the previous example that altered the second base of the ATG codon (Richards et al 2007), the mutation here altered the first base. Finally MLPA analysis identified a complete gene deletion in two patients (Table. 1).

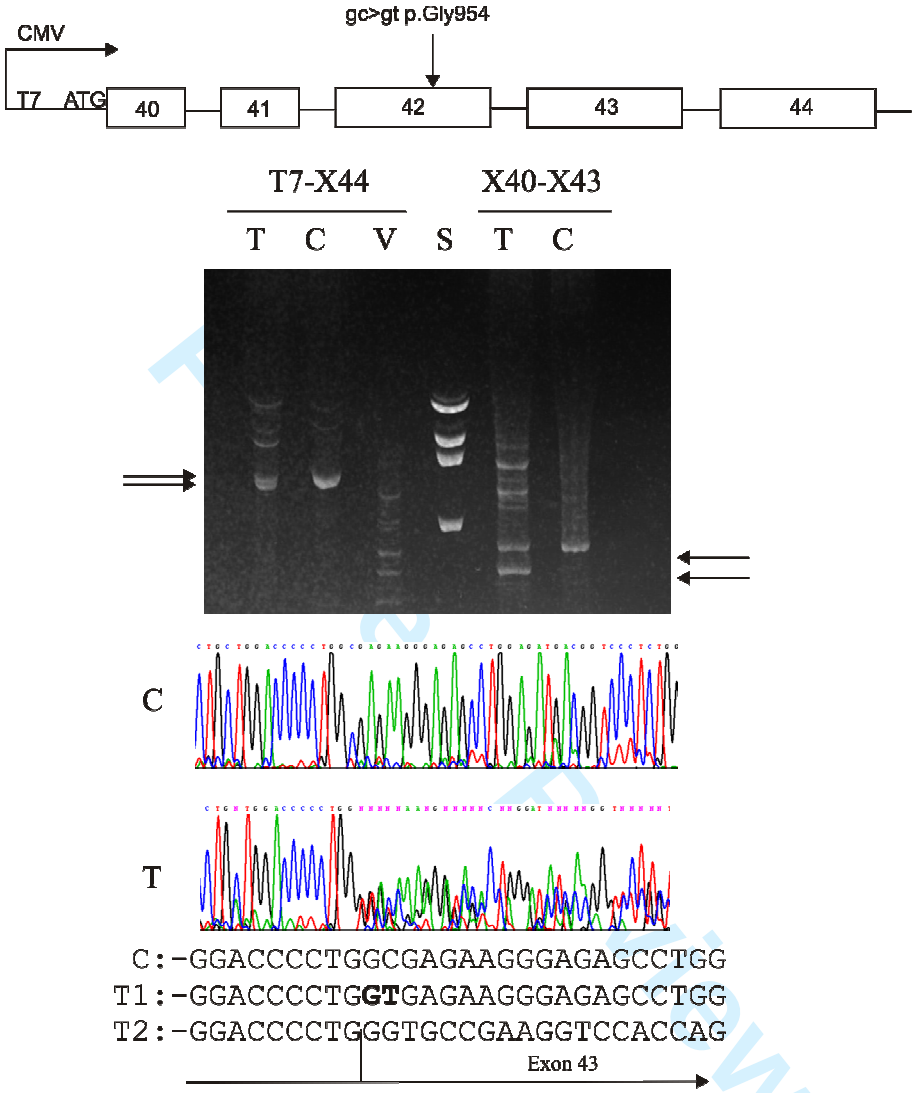

Figure 2. Splicing reporter analysis. Minigenes consisting of exons 40-44 and either the normal (C) or mutant (T) c.2862C>T, p.G954 alleles along with a vector control (V), were analysed by transfection into cultured cells and RT-PCR. Using primers corresponding to the vector T7 sequence and one in exon 44, a double band was observed for the T allele along with some slower migrating bands. Re-amplification with nested primers in exons 40 and 43 produced a similar profile but more clearly showed a deleted product from the T allele compared to the C allele (arrowed). The slower migrating bands are assumed to be heteroduplexes. Lane S are size standards, consisting of 400, 800,1200 and 2000bp. Sequencing showed that in addition to normal processing of the T allele, missplicing was occurring at the denovo GT site (in bold in sequence T1) created by the mutation, resulting in deletion of the final 35bp of exon 42 in the abnormally processed T2 sequence.

COL11A1 / Beaded vitreous phenotype

Since relatively few of the cases of Stickler syndrome with the beaded vitreous have been characterised, in some instances, when the family structure was suitable, linkage analysis was performed first, with markers for both

COL2A1 and *COL11A1*. *COL2A1* was excluded in all of these familial cases. Screening of *COL11A1* identified 13 mutations 12 were associated with the beaded phenotype. Eight were splice site mutations (Table. 1), and four had substitutions of glycine residues within the collagen triple helix. Where a cell line had been established the splice site mutations were analysed by RT-PCR, in all instances exon skipping was demonstrated.

No cell line was available for the IVS35+5G>A mutation, but over 180 normal chromosomes did not have this sequence change and they were not present in the SNP database. In one case MS307, vitreous assessment was not possible due to bilateral retinal detachments and the young age of the patient. Initial *COL2A1* screening was negative, but analysis of *COL11A1* identified a 27bp inframe deletion in exon 43, that also maintained the repeating Gly-Xaa-Yaa collagen sequence, which is also the case with skipping of collagen exons, that typically code for an amino acid sequence $[Gly\text{-}Xaa\text{-}Yaa]_n$ where n is often 18 and the exon is 54bp long.

Hypoplastic vitreous phenotype

Linkage analysis was also performed on a family (MS218) which did not display either the membranous or beaded vitreous phenotypes, but instead exhibited an empty hypoplastic vitreous gel on slit lamp examination. In this case *COL11A1* was excluded as the mutant locus. Screening of *COL2A1* then identified the causative mutation which was another novel glycine substitution p.G282D (Table. 1). In another case a sporadic patient MS344 was referred for gene screening without vitreous assessment. Analysis of *COL2A1* was negative but a mutation (triplication of T+2) of the donor splice site in intron 41 of *COL11A1* was found (Table. 1). Subsequent clinical examination found a similar hypoplastic vitreous phenotype. Both parents appeared clinically normal and molecular analysis confirmed that neither had the mutation.

Table 1 Mutations found in *COL2A1* and *COL11A1*

Mutation cDNA	Effect	Family	Examined	Vitreous Phenotype	Linkage
COL2A1					
Exon 1 c.1A>G	**p.M1>?**	**MS250**	1	Membranous	No
Exon 2 c.123T>A	**p.Y41X**	**MS58 PO STL1**	2	Membranous	No
Exon 2 **c.146delC**	**FS**	**MS49 PO STL1**	2	Membranous	No
Exon 2 c.166_167delGT	FS	MS226 PO STL1	4	Membranous	No
Exon 2 c.192C>A	p.C64X	MS319 PO STL1	1	Membranous	No
Exon 2 **c.211_233dup23**	**FS**	**MS298 PO STL1**	5	Membranous	No
IVS2 **c.293-1G>A** ASS ag>aa	**UDS**	**MS228**	4	Membranous	No
Exon 6 **c.406dupG**	**FS**	**MS208**	2	Membranous	No
Exon 7 **c.492delT**	**FS**	**MS236**	1	Membranous	No
Exon 7 **c.509dupC**	**FS**	**MS214**	1	Membranous	No
Exon 8 **c.572delC**	**FS**	**MS204**	3	Membranous	No
Exon 11 **c.719G>A**	**p.G240D (G40D)**	**MS137**	1	Membranous	No
Exon 11 **c.724delC**	**FS**	**MS186**	1	Membranous	No
Exon 11 **c.756dupT**	**FS**	**MS308**	2	Membranous	No
Exon 12 **c.808G>C**	**p.G270R (G70R)**	**MS121**	2	Membranous	No
Exon 13 **c.845G>A**	**p.G282D (G82D)**	**MS218**	3	**Hypoplastic**	**Yes**
IVS13 **c.870+1G>A** DSS gt>at	**UDS**	**MS89**	3	Membranous	No
Exon 14 **c.895_898delAAGG**	**FS**	**MS311**	1	Membranous	No
Exon 15 **c.930_933delGAGT**	**FS**	**MS139**	3	Membranous	No
Exon 16 **c.996_997delAG**	**FS**	**MS215**	2	Membranous	No
Exon 17 c.1030C>T	p.R344X (R144X)	MS88	3	Membranous	No
Exon 17 c.1030C>T	p.R344X (R144X)	MS312	2	Membranous	No
Exon 17 **c.1032delA**	**FS**	**MS191**	2	Membranous	No
IVS19 **c.1221+1G>C** DSS gt>gc	**UDS**	**MS245**	2	Membranous	No
IVS19 **c.1221+1delG** DSS gt> -t	**UDS**	**MS259**	1	Membranous	No
Exon 21 **c.1313delG**	**FS**	**MS244**	3	Membranous	No
Exon 21 **c.1358G>C**	**p.G453A (G253A)**	**MS306 SEDC**	1	Membranous	No

Exon 23 **c.1501G>C**	**p.G501R (G301R)**	**MS266 SEDC**	1	Membranous	No
IVS23 c.1527+135g>a CSS cgg>cag	Insertion of 234bp +PTC	MS147	4	Membranous	No
Exon 25 c.1597C>T	p.R533X (R333X)	MS283	2	Membranous	No
Exon 25 **c.1602dupG**	**FS**	**MS318**	1	Membranous	No
Exon 26 c.1693C>T	p.R565C (R365C)	MS56	8	Membranous	No
IVS27 c.1833+1G>A DSS gt>at	UDS	MS131	1	Membranous	No
IVS27 c.1833+1G>A DSS gt>at	UDS	MS295	3	Membranous	No
IVS27 c.1833+1G>A DSS gt>at	UDS	MS333	4	Membranous	No
IVS28 c.1888-1G>C ASS ag>ac	UDS	MS27	2	Membranous	No
Exon 29 **c.1908delT**	**FS**	**MS248**	1	Membranous	No
Exon 29 **c.1943dupG**	**FS**	**MS229**	3	Membranous	No
Exon 30 c.1957C>T	p.R653X (R453X)	MS145	7	Membranous	No
Exon 30 c.1957C>T	p.R653X (R453X)	MS252	1	Membranous	No
Exon 33 c.2101C>T	p.R701X (R501X)	MS268	1	Membranous	No
IVS33 **c.2194-1G>A** ASS ag>aa	**UDS**	**MS217**	1	Membranous	No
Exon 35 c.2353C>T	p.R785X (585X)	MS234	3	Membranous	No
Exon 38 **c.2473G>T**	**p.G825X (625X)**	**MS231**	1	Membranous	No
IVS 38 **c.2517+1G>C** DSS gt>ct	**UDS**	**MS106**	2	Membranous	No
IVS39 **c.2625+1G>T** DSS gt>tt	**UDS**	**MS128**	3	Membranous	No
Exon 40 **c.2653G>T**	**p.G885X (685X)**	**MS171**	2	Membranous	No
Exon 41 c.2710C>T	p.R904C (R704C)	MS321	1	Membranous	No
Exon 42 **c.2760delT**	**FS**	**MS184**	3	Membranous	No
Exon 42 **c.2787delA**	**FS**	**MS243**	1	Membranous	No
Exon 42 **c.2789delG**	**FS**	**MS313**	1	Membranous	No
Exon 42 c.2813dupC	FS	MS232	4	Membranous	No
Exon 42 **c.2814delT**	**FS**	**MS225**	2	Membranous	No
Exon 42 **c.2862C>T** CSS gcgaga>gtgaga	**p.G954 (G754)**	**MS122**	6	Membranous	No
Exon 42 **c.2862C>T** CSS gcgaga>gtgaga	**p.G954 (G754)**	**MS300**	1	Membranous	No
Exon 42 **c.2862C>T** CSS gcgaga>gtgaga	**p.G954 (G754)**	**MS287**	2	Membranous	No
Exon 42 **c.2869G>T**	**p.G957X (G757X)**	**MS323**	1	Membranous	No
IVS42 **c.2896-1G>A** ASS ag>aa	**UDS**	**MS272**	3	Membranous	No
Exon 43 **c.2976_2977delAG**	**FS**	**MS253**	1	Membranous	No
Exon/IVS43 **c.2974_3003+5del36** DSS	**UDS**	**MS144**	11	Membranous	No
IVS43 c.3003+1G>A DSS gt>at	UDS	MS235	1	Membranous	No
Exon 44 c.3106C>T	p.R1036X (R836X)	MS220	1	Membranous	No
Exon 44 c.3106C>T	p.R1036X (R836X)	MS249	5	Membranous	No
Exon 44 c.3106C>T	p.R1036X (R836X)	MS288	2	Membranous	No
Exon 45 c.3138delT	FS	MS325	2	Membranous	No
Exon 46 **c.3263_3264delGA**	**FS**	**MS135**	1	Membranous	No
Exon 49 **c.3474G>C**	**p.G1158A (G958A)**	**MS304 SEDC**	1	Membranous	No
Exon 49 **c.3488delG**	**FS**	**MS224**	3	Membranous	No
Exon 50 **c.3596dupC**	**FS**	**MS196**	3	Membranous	No
Exon 51 **c.3714C>A**	**p.Y1238X**	**MS219**	1	Uncertain	No
IVS51 c3886+1G>A DSS gt>at	Exon 51 skip	MS211	1	Membranous	No
IVS51 c3886+1G>A DSS gt>at	Exon 51 skip	MS263	4	Membranous	No
IVS51 c3886+2T>C DSS gt>gc	Exon 51 Skip	MS223	3	PO Membranous	No
Del Exons1-54	Gene Deletion	MS117	1	Membranous	No
Del Exons1-54	Gene Deletion	MS289	1	Membranous	No

COL11A1					
Intron 14 c.1630-2delA ASS	Exon 15 skip	MS92	3	Beaded	No
Intron 14 c.1630-2delA ASS	Exon 15 skip	MS111	6	Beaded	**Yes**
Intron 14 c.1630-2delA ASS	Exon 15 skip	MS246	3	Beaded	No
Intron 15 **c.1683+3A>C** DSS	**Exon 15 skip**	**MS140**	6	Beaded	No
Exon 16 **c.1694G>T**	**p.G565V (G37V)**	**MS107**	12	Beaded	**Yes**
Intron 18 **c.1845+5G>A** DSS	**Exon 18 skip**	**MS119**	3	Beaded	No
Intron 19 **c.1900-1G>A** ASS	**Exon 20 skip**	**MS216**	2	Beaded	**Yes**
Intron 22 **c.2043+1G>A** DSS	**UDS**	**MS193**	4	Beaded	**Yes**
Intron 35 **c.2755+5G>A** DSS	**UDS**	**MS238**	7	Beaded	**Yes**
Exon 40 **c.3079G>C**	**p.G1027R (G499R)**	**MS328**	1	Beaded	No
Intron 41 **c.3168+2T[3]** DSS	**UDS**	**MS344**	1	**Hypoplastic**	No
Exon 43 **c.3329_3355del27**	**p.V1110-P1118del (V582-P590)**	**MS307**	1	Uncertain	No
Exon 61 **c. 4538G>A**	**p.G1513D (G985D)**	**MS296**	1	Beaded	No
Exon 61 **c.4547G>T**	**p.G1516V (G988V)**	**MS316**	1	Beaded	No

COL2A1 Exons are numbered 1-54. Mutations are numbered in relation to the reference cDNA sequence NM_001844.3. This sequence has 157bp of a 5'untranslated region. +1 corresponds to the A of the ATG translation initiation codon. Amino acids are numbered corresponding to the reference sequence NP_001835.2. *COL11A1* Exons are numbered 1-6A,6B-67. Mutations are numbered in relation to the cDNA sequence NM-001854 that lacks exon 6B. +1 corresponds to the A of the ATG translation initiation codon. Amino acids are numbered corresponding to the reference sequence NP_001845.3.Amino acid substitutions within the collagen helix are also numbered (in parenthesis) according to the historical system whereby the first glycine of the collagen helix is 1. FS=frameshift. UDS=Undetermined splicing effect. ASS= acceptor splice site, DSS= donor splice site, CSS= cryptic splice site. Individuals /familes have type 1 stickler syndrome STL1, unless indicated, PO STL1= Predominantly ocular Stickler syndrome type 1. SEDC= spondyloepiphyseal dysplasia congenita. Linkage analysis performed prior to screening is also indicated. Complete gene deletions detected by MLPA are indicated by Del1-54. The vitreous phenotype was designated as membranous if one or more family members displayed this phenotype. The hypoplastic phenotype was designated if all affected individuals displayed the phenotype. The number of affected individuals clinically examined in each family is shown. Mutations in bold typeface represent novel mutations.

DISCUSSION

Although mutations resulting in Stickler syndrome are usually due to haploinsufficiency (Richards et al 2006) here we have identified three novel missense substitutions of glycine within the Gly-Xaa-Yaa repeat region of the type II collagen triple helix, namely p.G240D; p.G270R; p.G282D. Usually these dominant negative mutations result in more severe chondrodysplasias (Spranger et al 1994), and also demonstrated here in patients MS266, MS304 and MS306 that had SEDC. Two similar cases of glycine substitution resulting in mild phenotypes have previously been documented (Korkko et al 1993, Richards et al 2005), one was originally classified as Wagner syndrome p.G267D (G67D) and the other as dominantly inherited retinal detachment p.G318R (G118R). In those cases there was little or no involvement of non ocular tissues, Wagner syndrome was at that time frequently and erroneously considered as Stickler syndrome without systemic involvement. Here the Glycine substitutions resulted in typical Stickler syndrome with variable systemic features and indistinguishable from other patients with haploinsufficiency.

In comparison to type I collagen relatively few glycine substitutions in type II collagen have been documented. Factors that can affect the phenotype of osteogenesis imperfecta due to glycine substitutions in type I collagen include the nature of the substituting amino acid (Beck et al 2000, Marini et al 2007), disruption to intermolecular association with other components of the extracellular matrix (Marini et al 2007), and possible differences that the abnormal protein, retained within the endoplasmic reticulum, has on cell viability (Bateman et al 2009).

As indicated by the *COL2A1* silent mutations p.G654 (Richards et al 2007) p.G954, and the p.A107V substitution (Richards et al 2008) which create de novo splice sites, not all mutations may be having their effect via the amino acid change that missense mutations apparently result in but may also be affecting processing of pre-

mRNA. However previous analysis of the p.G318R mutation detected no abnormal splicing (Richards et al 2005). The location of these glycine substitutions, towards the N-terminal end of the collagen triple helix, is consistent with the observation that similarly located mutations in type I collagen resulted in almost exclusively non-lethal cases of osteogenesis imperfecta (Marini et al 2007) although non-lethal cases can also occur in other regions of the molecule. Too few of these missense *COL2A1* mutations have been fully characterised to draw firm conclusions regarding their pathogenic mode of action, as the effect of these apparent missense mutations on splicing, and on collagen synthesis, remains to be analysed.

Although there are no particular *COL2A1* hot spots, with mutations in virtually all of the 54 exons having been documented, some mutations such as the arginine to stop codon substitutions are recurrent. A proportion of these may be due to ancestral mutations, but others would appear to represent de novo mutations, based on the family structure. The splice site mutations found in *COL2A1* Stickler syndrome will presumably result in utilisation of cryptic splice sites leading to frame shifts and haploinsufficiency, as exon skipping would be expected to result in the more severe Kniest dysplasia (Wilkin et al 1999). However differences in the outcome of RNA processing may explain some of the phenotypic variability seen in Stickler syndrome (Richards et al 2008). We had previously suspected that one patient (MS117) lacked one *COL2A1* allele (Richards et al 2006). This was confirmed here by MLPA analysis that also detected a complete gene deletion in another patient. These two individuals represent only the second and third examples of a complete gene deletion of *COL2A1*. However the probes used for MLPA analysis are all within the *COL2A1* gene and so do not allow the extent of the genomic deletion to be determined.

To date only four glycine mutations have been documented in the collagen helix of α1(XI) collagen (Richards et al 1996, Annunen et al 1999, Majava et al 2007), this report doubled that number to eight which range from p.Gly565 to p.Gly1516. The position and type of substitutions within the collagen helix can affect the phenotype of various collagenopathies such as osteogenesis imperfecta (Marini et al 2007). Previously, mutations of *COL11A1* have either been described as resulting in Marshall syndrome, Stickler syndrome or of a mixed phenotype where it was difficult to distinguish between the two based on facial characteristics. Here all of the families / patients, except two displayed the beaded vitreous phenotype that we have previously associated with mutations in the *COL11A1* gene, emphasising the importance of vitreous assessment in the differential diagnosis. In contrast the systemic features associated with these *COL11A1* mutations were typical of the variability seen in Stickler syndrome.

In contrast to *COL2A1*, mutations so far documented in *COL11A1* have generally affected splice sites (Griffith et al 1998, Annunen et al 1999 Martin et al 1999, Poulson et al 2004 Majava et al 2007). Unlike *COL2A1*, where utilisation of cryptic splice sites are common, all of the splice site mutations in *COL11A1* that have been analysed by RT-PCR cause exon skipping. This results in inframe mRNAs and molecules capable of exerting a dominant negative effect via the association of mutant collagen chains with pro-alpha collagens from normal alleles of *COL11A1*, *COL11A2* or *COL2A1*. It is possible that haploinsufficiency of *COL11A1* results in a milder phenotype that is more difficult to recognise.

Previously the hypoplastic vitreous has been associated with unusual mutations in *COL2A1* p.L667F and p.G1305D (Richards et al 2000a, 2002), or seen sporadically within families that display the membranous phenotype (Richards et al 2006). Here we have seen it associated with mutations in both *COL2A1* and *COL11A1*, In the case of *COL11A1* (MS344), both parents were clinically and molecularly normal, and although the sequence chromatogram showed no evidence of mosaicism, we have seen the hypoplastic phenotype associated with mosaicism in *COL2A1*, however we have also seen it associated with a splicing mutation (Richards et al 2006). It is possible to confuse the congenital hypoplastic vitreous with developmental and progressive refractive related syneresis, so it is important not to dismiss a diagnosis of Stickler syndrome due to lack of the membranous or beaded phenotypes. Although the vitreous phenotype is a reliable indicator of mutant gene locus we suggest that it is used as a separate but complementary nomenclature to the “Stickler type 1, 2” etc. which has become synonymous with gene designation.

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NOMENCLATURE

Mutations annotated with c. and p. numbering correspond to guidelines suggested by HGVS (http://www.hgvs.org/mutnomen/). Mutations in parenthesis correspond to the historical system for collagens whereby the first glycine of the triple helix is numbered as 1.

REFERENCES

Ahmad NN, Ala-Kokko L, Knowlton RG, Jimenez SA, Weaver EJ, Maguire JI, Tasman W, Prockop DJ. 1991. Stop codon in the procollagen II gene (COL2A1) in a family with the Stickler syndrome (arthro-ophthalmopathy). Proc Natl Acad Sci USA 88:6624-6627.

Annunen S, Korkko J, Czarny M, Warman ML, Brunner HG, Kaariainen H, Mullikan JB, Tranebjarg L, Brooks DG, Cox GF, Cruysberg JR, Curtis MA, Davenport SL, Friedrich CA, Kaitila I, Krawczynski MR, Latos-Bielenska A, Mukai S, Olsen BR, Shinno N, Somer M, Vikkula M, Zlotogora J, Prockop DJ, Ala-Kokko L. 1999. Splicing mutations of 54-bp exons in the COL11A1 gene cause Marshall syndrome, but other mutations cause overlapping Marshall / Stickler phenotypes. Am J Hum Genet 65:974-983.

Bateman JF, Boot-Handford RP, Lamande SR. 2009. Genetic diseases of connective tissues:cellular and extracellular effects of ECM mutations, Nat Rev Genet 10:173-183.

Beck K, Chan VC, Shenoy N, Kirkpatrick A, Ramshaw JA, Brodsky B. 2000. Destabilization of osteogenesis imperfecta collagen-like model peptides correlates with the identity of the residue replacing glycine. Proc Natl Acad Sci USA 97:4273-4278.

Blaschke UK, Eikenberry EF, Hulmes DJ, Galla HJ, Bruckner P. 2000. Collagen XI nucleates self-assembly and limits lateral growth of cartilage fibrils. J Biol Chem 275:10370-10378.

Brunner HG, van Beersum SEC, Warman ML, Olsen BR, Ropers H-H, Mariman ECM. 1994. A Stickler syndrome gene is linked to chromosome 6 near the COL11A2 gene. Hum Mol Genet 3:1561-1564.

Griffith AJ, Sprunger LK, Sirko-Osadsa A, Tiller GE, Meisler MH, Warman ML. 1998. Marshall syndrome associated with a splicing defect at the COL11A1 locus. Am J Hum Genet 62:816-823.

Körkkö J, Ritvaniemi P, Haataja L, Kääriäinen H, Kivirikko KI, Prockop DJ, Ala-Kokko L. 1993. Mutation in type II procollagen (COL2A1) that substitutes aspartate for glycine alpha 1-67 and that causes cataracts and retinal detachment: evidence for molecular heterogeneity in the Wagner syndrome and the Stickler syndrome (arthro-ophthalmopathy) Am J Hum Genet 53:22-25.

Marini JC, Forlino A, Cabral WA, Barnes AM, San Antonio JD, Milgrom S, Hyland JC, Körkkö J, Prockop DJ, De Paepe A, Coucke P, Symoens S, Glorieux FH, Roughley PJ, Lund AM, Kuurila-Svahn K, Hartikka H, Cohn DH, Krakow D, Mottes M, Schwarze U, Chen D, Yang K, Kuslich C, Troendle J, Dalgleish R, Byers PH. 2007. Consortium for osteogenesis imperfecta mutations in the helical domain of type I collagen: regions rich in lethal mutations align with collagen binding sites for integrins and proteoglycans. Hum Mut 28:209-221.

Marjava M, Hoornaert KP, Bartholdi D, Bouma MC, Bouman K, Carrera M, Devriendt K, Hurst J, Kitsos G, Niedrist D, Petersen MB, Shears D, Stolte-Dijkstra I, Van Hagen JM, Ala-Kokko L, Mannikko M, Mortier GR. 2007. A report on 10 new patients with heterozygous mutations in the COL11A1 gene and a review of genotype-phenotype correlations in type XI collagenopathies. Am J Med Genet 143A:258-264.

Martin S, Richards AJ, Yates JRW, Scott JD, Pope FM, Snead MP. 1999. Stickler Syndrome: further mutations in COL11A1 and evidence for additional locus heterogeneity. Eur J Hum Genet 7:807-814.

Meredith SP, Richards AJ, Bearcroft P, Pouson AV, Snead MP. 2007. Significant ocular findings are a feature of heritable bone dysplasias resulting from defects in type II collagen. Br J Ophthalmol 91:1148-1151.

Poulson AV, Hooymans JM, Richards AJ, Bearcroft P, Murthy R, Baguley DM, Scott JD, Snead MP. 2004. Clinical features of type 2 Stickler syndrome. J Med Genet 41:e107.

Richards AJ, Yates JRW, Williams R, Payne SJ, Pope FM, Scott JD, Snead MP. 1996. A family with Stickler syndrome type 2 has a mutation in the COL11A1 gene resulting in the substitution of glycine 97 by valine in □1(XI) collagen. Hum Mol Genet 5:1339-1343.

Richards AJ, Baguley DM, Yates JR, Lane C, Nicol M, Harper PS, Scott JD, Snead MP. 2000a. Variation in the vitreous phenotype of Stickler syndrome can be caused by different amino acid substitutions in the X position of the type II collagen Gly-X-Y triple helix. Am J Hum Genet 67:1083-1094.

Richards AJ, Martin S, Yates JRW, Scott JD, Baguley DM, Pope FM, Snead MP. 2000b. COL2A1 exon 2 mutations: relevance to the Stickler and Wagner syndromes. Br J Ophthalmol 84:364–371.

Richards AJ, Morgan J, Bearcroft PW, Pickering E, Owen MJ, Holmans P, Williams N, Tysoe C, Pope FM, Snead MP, Hughes H. 2002. Vitreoretinopathy with phalangeal epiphyseal dysplasia, a type II collagenopathy resulting from a novel mutation in the C-propeptide region of the molecule. J Med Genet 39:661-665.

Richards AJ, Meredith S, Poulson A, Bearcroft P, Crossland G, Baguley DM, Scott JD, Snead MP. 2005. A novel mutation of COL2A1 resulting in dominantly inherited rhegmatogenous retinal detachment. Invest Ophthalmol Vis Sci 46:663-668.

Richards AJ, Laidlaw M, Whittaker J, Treacy B, Rai H, Bearcroft P, Baguley DM, Poulson A, Ang A, Scott JD, Snead MP. 2006. High efficiency of mutation detection in type 1 stickler syndrome using a two-stage approach: vitreoretinal assessment coupled with exon sequencing for screening COL2A1. Hum Mut 27:696-704.

Richards AJ, Laidlaw M, Meredith SP, Shankar P, Poulson AV, Scott JD, Snead MP. 2007. Missense and silent mutations in COL2A1 result in Stickler syndrome but via different molecular mechanisms. Hum Mut 28:639.

Richards AJ, Snead MP. 2008. The influence of pre-mRNA splicing on phenotypic modification in Stickler's syndrome and other type II collagenopathies. Eye 22:1243-1250.

Snead MP, Yates JRW. 1999. Clinical and Molecular Genetics of Stickler syndrome. J Med Genet 36:353-9.

Spranger J, Winterpacht A, Zabel B. 1994. The type II collagenopathies: a spectrum of chondrodysplasias. Eur J Pediatr 153:56-65.

Vikkula M, Mariman ECM, Lui VCH, Zhidkova NI, Tiller GE, Goldring MB, van Beersum SEC, de Waal Malefijt MC, Vandenhoogen FHJ, Ropers HH, Mayne R, Cheah KSE, Olsen BR, Warman ML, Brunner HG. 1995. Autosomal dominant and recessive osteochondrodysplasias associated with the COL11A2 locus. Cell 80:431-437.

Wilkin DJ, Artz AS, South S, Lachman RS, Rimoin DL, Wilcox WR, McKusick VA, Stratakis CA, Francomano CA, Cohn DH. 1999. Small deletions in the type II collagen triple helix produce kniest dysplasia. Am J Med Genet 16:105-112.