A Novel Mutation of COL2A1 Resulting in Dominantly Inherited Rhegmatogenous Retinal Detachment

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PURPOSE. To determine the molecular defect in a family with autosomal dominant rhegmatogenous retinal detachment (DRRD), and to investigate missplicing as a possible phenotypic modifier of mutations in COL2A1.

METHODS. Clinical examination of the family and linkage analysis using markers flanking COL2A1 and COL11A1, the known loci for Stickler syndrome; mutation screening of COL2A1; construction of splicing reporter minigenes and transfection into cultured cells; and RT-PCR analysis of reporter specific transcripts.

RESULTS. A family with DRRD showed no systemic clinical signs (skeletal, orofacial, or auditory) usually associated with Stickler syndrome. Linkage analysis excluded COL11A1 as the disease locus but could not exclude COL2A1. Mutation screening of COL2A1 identified a novel G118R mutation in type II collagen. Transfection of minigenes carrying mutations associated with DRRD (G118R, R453X, and L467F) into cultured cells detected no missplicing of mRNA from mutant constructs.

CONCLUSIONS. Mutations outside the alternatively spliced exon 2 region of COL2A1 can also result in an ocular only phenotype. There was no evidence that missplicing modifies the phenotype of these mutations, suggesting that the minimal or absent systemic features demonstrated by the G118R and L467F mutations are the result of the biophysical changes imparted on the collagen molecule. (Invest Ophthalmol Vis Sci. 2005;46: 663–668) DOI:10.1167/iovs.04-1017

Rhegmatogenous retinal detachment (RRD) most frequently results from retinal tearing at the time of posterior vitreous detachment. Nonsyndromic RRD can be inherited in a clearly dominant fashion, although in most of these cases, the genetic locus for the disorder is unknown. However, RRD is also a common feature of the type II collagenopathies (disorders due to mutations in the gene for type II collagen, COL2A1) and some recent examples of mutations in this gene suggest that COL2A1 should be considered a candidate gene for dominant RRD (DRRD).

Type II collagen is a major component of the vitreous and cartilage. Mutations in COL2A1 can lead to a wide spectrum of dominant disorders affecting both tissues. The most severe type II collagenopathies are achondrogenesis II and hypochondrogenesis (Mendelian inheritance in man [MIM] 200610), which are perinatally lethal. Other phenotypes are spondylo-epiphyseal dysplasia congenita (SED; MIM 183900), Kniest dysplasia (MIM 156500) and spondyloepimetaphyseal dysplasia (SEMD; MIM 184250), a group of disproportionate short stature syndromes that have variation in the presentation of radiographic features. These five phenotypes usually result from dominant negative mutations that produce abnormal (αII) collagen capable of coassembly with collagen produced from the normal allele. If expressed in a 1:1 ratio these results in one of eight normal and seven of eight mutant type II collagen homotrimers. The most frequent dominant negative mutations in collagen genes are those that lead to missense substitutions of obligate glycines in the repeating Gly-Xaa-Yaa collagen helix. It has long been postulated that the resultant phenotype in the fibrillar collagenopathies (types I, II, III, V, and X) is at least partly dependent on the nature of the substituting amino acid its position in the molecule and how the change may alter the posttranslational modification and biophysical character of the collagen and resulting collagen fibrils. Nevertheless, the difficulty in predicting the outcome from the amino acid change alone suggests that other factors are involved.

The most common type II collagenopathy is type I Stickler syndrome (STL1, MIM 108300). This is a comparatively milder disorder usually resulting from haploinsufficiency of type II collagen. Affected individuals usually have myopia, have a high incidence of retinal detachment, can have premature osteoarthritis, and may have some degree of deafness and orofacial dysmorphology. Nearly all of STL1-affected individuals have mutations that result in premature termination codons. These may be subject to nonsense-mediated decay (NMD), and mRNA quality control surveillance mechanism that degrades mutant mRNAs with premature termination codons. Virtually all patients with STL1 have a vestigial vitreous gel in the retrolental space, bounded by a membrane: the type I membrane anomaly. This helps to distinguish these from other patients with Stickler syndrome type 2 (STL2, MIM 604841) who have mutations in COL11A1 and a different vitreous phenotype.

Affected individuals in families with DRRD display neither of the vitreous phenotypes recognized in the Stickler syndromes and show no signs of skeletal dysplasia or deafness. In most cases, the genetic basis for this disorder is unknown, but three examples suggest that mutations in COL2A1 may be involved. We described the first, a family with atypical Stickler syndrome and an unusual L467F mutation in type II collagen, with optically empty vitreous and minimal or completely absent systemic features. The second was a family with a novel mutation in the C-propeptide region of the molecule, exhibiting a vitreous architecture similar to that seen in cases of DRRD, with the exception that they also had skeletal dysplasia features. The third was a family with DRRD, described by Go et al., with an R453X mutation, but no systemic features; an
identical mutation has been described in a case of Stickler syndrome.\textsuperscript{1,2} We have also seen this mutation in two unrelated cases of Stickler syndrome (described later). As Stickler syndrome has a common pathogenic mechanism (haploinsufficiency), the phenotypic variability in this disorder, suggests that other modifying factors affect the clinical appearance. Recently, it has become apparent that missense or nonsense mutations can affect exon splicing enhancers, resulting in altered splicing of mutant transcripts,\textsuperscript{13–17} and the degree to which the mutant allele is misspliced can alter the phenotypic outcome.

Herein, we characterize a novel mutation in \textit{COL2A1} resulting in DRRD, and investigate missplicing as a potential phenotypic modifier of missense and nonsense mutations associated with DRRD.

**METHODS**

Pedigrees were identified from the vitreous research clinic at Addenbrooke’s hospital, and studies were performed with approval of the local ethics committee (LREC 92/019 and 02/172). Informed written consent was obtained in all cases, and the protocol adhered to the tenets of the Declaration of Helsinki.

Ophthalmic, orofacial, skeletal, and auditory features were assessed using the methods reported previously.\textsuperscript{5} 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 performed, including slit lamp biomicroscopy and indirect ophthalmoscopy with scleral depression. In some of the younger patients,planationometry and gonioscopy were not possible. Anterior and posterior segment photographs were taken when appropriate. Joint hypermobility was assessed objectively using the Beighton scoring system.

All patients underwent bilateral otoscopy and audiometry involving air and bone conduction testing according to standardized procedures.

**Clinical Descriptions**

**Family MS4.** The proband was one of four affected individuals in a three-generation family and presented with bilateral giant tear detachments at the age of 14 years. All affected individuals had myopia, exhibiting the membranous vitreous anomaly typical of type 1 Stickler syndrome. The proband underwent bilateral retinal detachment repair with vitrectomy and internal tamponade. The other affected members were treated prophylactically with 360° contiguous retinal cryotherapy and their retinas have remained attached through 13 years of follow-up. Two individuals had high, arched palates, and all patients exhibited joint laxity. Radiologic changes were characterized by minor scoliotic changes, anterior vertebral wedging, and osteophytosis.

**Family MS25.** The family has been described previously\textsuperscript{1} and had an atypical form of Stickler syndrome/DRRD.

**Family MS31.** The index case was the daughter of unaffected parents and had one affected son. Both had the membranous vitreous anomaly and systemic features of STL1.

**Family MS68.** Nine members of a large five-generation family were examined. Although a single patient exhibited high myopia, refractive error in affected individuals was generally minimal, ranging from emmetropia to moderate myopia, with vitreous architecture consistent with their age and refractive error. No individual exhibited the congenital vitreous anomalies usually present in the Stickler syndromes, and most exhibited patchy, circumferential lattice retinopathy in contrast to the paravascular distribution characterized by Stickler syndrome. There was a high incidence of retinal detachment but no evidence of systemic involvement such as premature arthropathy, cleft palate, or hearing loss. The family disorder was classified as DRRD.

**Family MS118.** The index case was a member of a three-generation family with at least four affected individuals. All those examined had the type 1 membranous vitreous anomaly and systemic features of STL1. The proband had moderate myopia, high-frequency sensorineural hearing loss, maxillary hypoplasia, micrognathia, and severe premature arthropathy, culminating with hip replacement surgery at the age of 35 years.

**Linkage Analysis**

The DNA from nine members of MS68 was used to amplify polymorphic markers close to \textit{COL2A1} (D12S85, D12S361, and \textit{COL2A1}: 5 VNTR) and \textit{COL11A1} (D15248, D152626, and D15495), as previously described.\textsuperscript{2} Two-point linkage analysis was performed using MLINK.\textsuperscript{19}

**Mutation Screening**

Mutation detection in \textit{COL2A1} was essentially as previously described.\textsuperscript{18}

**Splicing Reporter Construction**

Genomic regions of \textit{COL2A1} beginning and ending with complete exons were amplified from affected individuals, by using primers that introduced an \textit{EcoR}\textit{I} and a \textit{KpnI} restriction enzyme site at the 5’ and 3’ end of the minigene, respectively. In addition, a Kozak translation initiation sequence (\textit{GCCATGG}) was inserted immediately before the first exon of the minigene, which in each case started with the last nucleotide (G) of the Kozak sequence (Table 1). Amplification of DNA was then performed (Pfu Turbo; Stratagene, La Jolla, CA). Typically, genomic DNA was amplified in a 100-μL reaction volume, using 5 U of enzyme in the buffer supplied by the manufacturer, 400 μM dNTPs, and 25 picomoles of each primer. After an initial denaturation at 95°C
Table 2. Mutation Phenotype Results

<table>
<thead>
<tr>
<th>Family</th>
<th>Disorder</th>
<th>Vitreous Phenotype</th>
<th>Mutation</th>
<th>Missplice</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS4</td>
<td>Stickler syndrome</td>
<td>Membranous anomaly</td>
<td>g.20992G→T</td>
<td>Exon 51 skip</td>
</tr>
<tr>
<td>MS25</td>
<td>Stickler syndrome/DRRD</td>
<td>Optically empty vitreous</td>
<td>LV581+1G→T, p.L667F (L467F), g.20996G→T</td>
<td>Normal</td>
</tr>
<tr>
<td>MS31</td>
<td>Stickler syndrome</td>
<td>Membranous anomaly</td>
<td>R653X (R453X), g.20711C→T</td>
<td>ND</td>
</tr>
<tr>
<td>MS68</td>
<td>Dominant RRD</td>
<td>Disorganised architecture</td>
<td>G318R (G118R), 10858G→A</td>
<td>Normal</td>
</tr>
<tr>
<td>MS118</td>
<td>Stickler syndrome</td>
<td>Membranous anomaly</td>
<td>R653X (R453X), g.20711C→T</td>
<td>ND</td>
</tr>
</tbody>
</table>

Missense mutations are described as amino acid changes according to the protein reference sequence (NP_001835) and also using the historical numbering system whereby amino acids are numbered from the first glycine of the type II collagen helix (in parentheses). Nucleotide substitutions are numbered according to the complete gene sequence (accession no. L10547). The COL2A1 exons are numbered 1 to 54. In addition to the nucleotide number, the intron 51 mutation is numbered from the first nucleotide of the intron. The effect of the mutations on splicing was determined by RT-PCR using RNA from cultured skin fibroblasts. ND, not determined.

for 5 minutes, 40 cycles each of 95°C for 1 minute, 65°C for 1 minute, and 72°C for 3 minutes were used to amplify the DNA. Amplified products were cleaned with PCR purification spin columns (Qiagen; Qiagen, Valencia, CA), eluted in water, and incubated sequentially with EcoRI and then KpnI to generate cohesive ends. The digested product was then ligated into the expression vector pCDNA3.1 version A (Invitrogen, Carlsbad, CA), so that correctly spliced transcripts were in frame with the plasmid encoded c-myc epitope and the poly His tag. Ligated DNA was then transformed into competent Escherichia coli strain DH5α. Transformants were sequenced to identify normal and mutant clones and to ensure that no additional nucleotide substitutions had been introduced by amplification or cloning.

Cell Lines and Tissue Culture

Primary cell cultures were established from corneal and scleral tissue after enucleation of an eye for end-stage glaucoma. The transformed cell line SW1355 was purchased from the American Type Culture Collection (Manassas, VA), and the OUMS-27 cells were obtained from the Japanese Health Sciences Foundation (No. IP050488). An osteoarthritis cell line Saos-2 was a gift from Tony Kouzandres (Wellcome Trust/Cancer Research UK, Gordon Institute, Cambridge, UK), an immortal Müller cell line, MIO-M1, was a gift from Astrid Limb (Institute of Ophthalmology, London, UK). All these were grown as monolayer cultures on plastic dishes in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U penicillin and 100 µg/mL streptomycin, 1 mM nonessential amino acids (Invitrogen), and 25 µg/mL ascorbic acid. Except MIO-M1 cells, which were grown under the same conditions but in DMEM with cell culture medium (GluTamax 1; Invitrogen), 10% FBS, and antibiotics and other supplements as just listed.

Transfection

Cells were transfected with minigene constructs using a lipophilic transfection reagent (Lipofectamine; Invitrogen), and 10 µg of DNA was allowed to complex with the transfection reagent in 1.5 mL serum-free medium. Cells at between 80% and 90% confluence, in a 100-mm diameter culture dish, were washed twice with serum-free medium and incubated with the DNA/transfection reagent complex in a total volume of 6.5 mL of serum-free medium. After 4 hours, the culture medium was replaced with medium containing FBS and incubated for 24 hours before isolation of RNA. For certain experiments cells were also incubated with 300 µg/mL emetine, to inhibit nonsense-mediated decay.

Reverse Transcription–Polymerase Chain Reaction

 Cultured cells were harvested by trypsinization and RNA extracted, by using shredder columns (QIAshredder column; Qiagen) to lys the cells and purification spin columns (RNaseasy; Qiagen) for preparation of RNA. Illegitimate transcripts were amplified as previously described, with gene-specific primers for the reverse transcription reaction as well as the PCR. For RNA obtained from transfection experiments, endonuclease digestion with RNase-free DNase I was used to remove trace amounts of cloned DNA. Approximately equal amounts (4 µg) of this RNA were then used to amplify cDNA, a vector-specific primer (BGHR) was used for the reverse transcription reaction, and a gene-specific antisense primer, along with a vector specific sense primer (T7), was used for amplification.

RESULTS

The familial disorders were classified on the basis of vitreoretinal phenotypes and the systemic features displayed (Table 2). Those with the typical type 1 membranous anomaly and arthropathy and/or deafness, and/or cleft palate were designated as Stickler syndrome. Family MS68 did not have either the membranous or beaded vitreous phenotypes associated with Stickler syndrome, or any systemic features. They were therefore classified as having an inherited form of RRD. All individuals that had an abnormal vitreoretinal appearance were classed as affected regardless of whether they had experienced a detachment, which would only be evident after separation of the posterior hyaloid membrane. Family MS68 was analyzed for linkage to either COL2A1 or COL11A1, by using polymorphic markers flanking each gene. Whereas COL11A1 was excluded as the disease locus, COL2A1 could not be excluded. A lod score of 1.01, at zero recombination, was obtained for the nine family members tested, using the COL2A1 VNTR marker. Mutation screening of COL2A1 detected mutations in all cases (Table 2, Fig. 1). Historically, amino acid substitutions in collagen molecules are numbered from the first glycine of the collagen triple helix. However, this is at odds with current thinking that the most complete reference sequence should be used (http://www.hgvs.org/mutnomen/). To compare mutations described herein with previously described mutations, we used both nomenclatures (Table 2).

The family with DRRD (MS68) had a novel mutation leading to the substitution of glycine within the collagen helix. All affected members of the family had the mutation, whereas unaffected individuals had only the normal sequence. Usually, this type of dominant negative mutation results in severe phenotypes such as SEDC and SEMD. Two families with Stickler syndrome (MS31 and MS118) had identical mutations leading to a premature termination codon (R453X). This mutation has been seen in a previous example of Stickler syndrome and...
also a family with DRRD. Family MS4 had a donor splice site mutation that altered the first nucleotide of intron 51. Amplification of illegitimate transcripts using RNA from patients’ cultured cells did not indicate any missplicing events for either of the missense mutations. The G3T mutation in the donor splice site of intron 51 resulted in skipping of exon 51. This caused a frameshift and a premature termination codon in exon 52, 140 nucleotides upstream from the exon 52–intron 52 splice site. To determine whether the G118R, L467F, or R453X mutations affect splicing in specific cell types, mini-genes containing the mutations (Fig. 2) were transfected into cultured cells derived from either ocular or skeletal tissue. No abnormal RT-PCR products were observed when the 118R or the 453X mutations were transfected into any of the cell lines used (Fig. 3). In addition inhibition of NMD in MIO-M1 and Saos-2 cells transfected with the G118R mutation did not result in detection of misspliced RT-PCR products (data not shown). A quantitatively minor product was observed when the 467F mutant was transfected into all cell lines (Fig. 3). However, when this product was gel purified and reamplified, it produced the same banding profile as the original RT-PCR. Sequencing of the gel-purified cDNA resulted in only the single base pair mutant cDNA sequence with no evidence of missplicing (data not shown). It was therefore concluded that the aberrant band was probably a conformational form of the mutant cDNA that migrated more slowly in the polyacylamide gel. In contrast to the exonic mutations, the mutation affecting the donor splice site of intron 51 was misspliced when transfected into all cell lines (data only shown for MIO-M1; Fig. 3).

**DISCUSSION**

Mutations of COL2A1 usually result in both a skeletal and ocular phenotype, with a characteristic vestigial vitreous gel in the retrolental space bounded by a membrane (type 1 membranous phenotype). This vitreous phenotype was instrumental in identifying a group of patients with no systemic features of Stickler syndrome but an identical eye phenotype. We classified these as a predominantly ocular form of Stickler syndrome and identified mutations in the alternatively spliced exon 2 of the COL2A1 gene. This observation has been confirmed by others, although Gupta et al confused this phenotype with Wagner’s vitreoretinal degeneration (MIM...
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143200), which has a different vitreoretinal appearance and a lower incidence of retinal detachment.28–30

Families with dominantly inherited retinal detachment also have no systemic features and do not have either the membranous or beaded vitreous phenotypes associated with Stickler syndromes 1 and 2. Consequently, assignment of genetic loci that cause this disorder relies on linkage analysis of large families. Only two similar families have been fully characterized. One had an overlapping Stickler/DRRD phenotype,3 whereas the other had a mutation identical with that in cases of Stickler syndrome.3,12 and some of these family members had a membranous vitreous phenotype.3 In the present study we characterized a novel mutation in COL2A1 resulting in DRRD. Surprisingly, it changes an obligate glycine in the collagen helix to arginine. This type of dominant negative mutation usually results in severe chondrodysplasia, because the mutant protein coassembles with normal collagen, affecting its posttranslational modification and secretion.5–7 However, the phenotypic outcome of these changes is difficult to predict and may depend on the nature of the amino acid change and its position within the collagen molecule. Unlike the severe type II collagenopathies, there appears to be a common molecular mechanism for nearly all cases of Stickler syndrome. Premature termination codons lead to nonsense-mediated decay and haploinsufficiency. However, even Stickler syndrome can present with a wide phenotypic spectrum, suggesting that other modifying factors are involved.

Recently, it has become apparent that missplicing resulting from missense or nonsense mutations that affect exon splicing enhancers may be one factor that can modify disease phenotype.13–17 Splicing of mRNA can involve enhancers of the GT-AG donor-acceptor splice sites at the exon-intron boundaries as well as silencers. These enhancer and silencer sequences recruit transacting factors that either promote or inhibit recognition of potential splice sites by the spliceosome and help to differentiate correct donor and acceptor splice sites from similar sequences that can be found throughout the genome. It has become clear that some missense pathogenic mutations actually have their effect, not solely through the amino acid substitution, but by altering an exon splicing enhancer sequence and causing exon skipping, as well.13–17 This also explains how some nonsense mutations result in exon skipping and in-frame transcripts, rather than nonsense mediated decay.13–15 Not only can variation in the efficiency of splicing explain tissue specific phenotypes, but differences in these transacting splicing factors may also explain clinical variability between individuals with identical mutations.31–33

If an exonic mutation alters the normal splicing profile of an mRNA, it may have a striking effect on collagen mutations, where missense mutations tend to have severe phenotypes and nonsense mutations milder outcomes. For instance, when a mutation results in a premature termination codon, the most transcripts may be subject to nonsense-mediated decay. However, if a small proportion were misspliced (exon skipped), due to disruption of an exon-splicing enhancer, the resultant shortened collagen molecules would exert a dominant negative effect (most collagen gene exons code for complete codons) and make the phenotype more severe. Conversely any missplicing resulting from a missense mutation that produced transcripts with a premature termination signal would reduce the severity of the phenotype by reducing the amount of mutant protein capable of exerting a dominant negative effect. We therefore examined whether missense and nonsense mutations associated with DRRD affects splicing. Because affected tissue is difficult to obtain from these patients we first analyzed illegitimate transcripts. Second, we created splicing reporter constructs. These consisted of minigenes containing regions of COL2A1 with the mutant or normal sequence in a mammalian expression vector. When these were transfected into a variety of cell lines from both ocular and skeletal tissues, only normally spliced transcripts were detected. This was in contrast to a mutation that altered the donor splice site of intron 51, where exon skipped mRNAs were detected in both illegitimate transcripts and RNA from transfected cell lines. We therefore have found no evidence that missplicing of these mutations modifies

FIGURE 3. Splicing reporter analysis of G118R, L467F, R453X, and IVS51+1G→T mutations. Minigene constructs (Fig. 2) containing either the normal or altered sequence of COL2A1 mutations were transfected into cultured cells as indicated. Products from RT-PCRs performed using RNA extracted from transfected cells were analyzed by electrophoresis in 5% polyacrylamide gels along with a standard 100 bp DNA ladder (M). Arrow: a quantitatively minor aberrant band seen in cDNA isolated from cells transfected with the 467F mutation is shown in the panel showing MIO-M1 cells. Transcripts amplified from cDNA isolated from cells transfected with the 467F mutation is shown in the panel showing MIO-M1 cells. Transcripts amplified from construct C that contain exon 51 (+) or skip exon 51 (−) are marked.
the resultant phenotype. At present, it appears that the G118R and L467F mutations disrupt the collagen helix and/or fibril formation less than other missense mutations that result in severe phenotypes, such as achondrogenesis II, SEDC, or SEMD. However, it is still unclear why the family with the R453X mutation reported by Go et al. had none of the systemic features usually associated with Stickler syndrome. Perhaps, as suggested by those investigators, other genetic factors modified the phenotype in that family, possibly including splicing factors not expressed in the cell cultures used in this study.

Whatever the explanation for these phenotypically milder examples, it is now apparent that, in COL2A1, mutations can result in ocular-only phenotypes, even when the mutation lies outside the exon 2 region of the gene. This has implications for clinical diagnosis and genetic screening protocols for individuals and families with vitreoretinal disorders.

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References


