



Alport syndrome: impact of digenic inheritance in patients management

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The impact of non-Mendelian inheritance in Alport syndrome

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Abstract

Alport syndrome (ATS) is a genetically heterogeneous nephropathy with considerable phenotypic variability and different transmission patterns, including Mendelian (X-linked/autosomal) and non-Mendelian inheritance (digenic). Here we present a new series of families with digenic inheritance and we discuss consequences for genetic counseling and risk assessment. Out of 5 families harboring variants in more than one *COL4* gene detected by Next Generation Sequencing (NGS), minigene splicing assay allowed us to identify four as true digenic. Two families showed *COL4A3/A4* mutations *in cis*, mimicking an autosomal dominant inheritance with a more severe phenotype and one showed *COL4A3/A4* mutations *in trans*, mimicking an autosomal recessive inheritance with a less severe phenotype. In a fourth family a *de novo* mutation (*COL4A5*) combined with an inherited mutation (*COL4A3*) triggered a more severe phenotype. A fifth family, predicted digenic on the basis of silico tools, rather showed monogenic X-linked inheritance due to a hypomorphic mutation, in accordance with a milder phenotype. In conclusion, this study highlights the impact of non-Mendelian inheritance in ATS and explains the associated atypical presentations. More complex inheritance should be therefore considered when reviewing prognosis and recurrence risks. On the other side, these findings emphasize the importance to accompany NGS with splicing assays in order to avoid erroneous identification of at risk members.

Key words

Alport syndrome, digenic inheritance, hypomorphic allele, massive parallel sequencing.

INTRODUCTION

Alport syndrome (ATS) is a clinically heterogeneous nephropathy accounting for 0.3-2.3% of patients who develop end-stage renal disease (ESRD) (1). It is caused by mutations in collagen IV genes encoding the alpha 3-4-5 heterotrimer, which is produced by podocytes and represents the major constituent of the mature glomerular basement membrane (GBM) (2). In ATS, failure to substitute the immature form (alpha 1-2-1 chains) of collagen IV with the alpha 3-4-5 chains results in progressive kidney injury and ultimately ESRD, associated with glomerular sclerosis and tubulo-interstitial fibrosis, inflammation and reorganization of the extracellular matrix (1). Specific ultrastructural lesions of the GBM include: irregular thinning, widespread thickening with splitting and fragmentation of the lamina densa (1). Affected patients often manifest additional extra-renal manifestations, such as bilateral high-tone sensorineural hearing loss and pathognomic ocular lesions (3,4).

Three classic models of Mendelian inheritance have been reported in ATS: semidominant X-linked (XLAS; MIM#301050) due to mutations in *COL4A5* (Xq22.3), autosomal recessive (ARAS; MIM#203780) caused by mutations in *COL4A3* or *COL4A4* genes, located head-to-head on chromosome 2 (2q36-37) and autosomal dominant (ADAS; MIM#104200) (5-11). The X-linked semidominant form, accounting for the majority of cases, is clinically characterized by severely affected males with persistent hematuria, proteinuria, with inevitable progression to ESRD (typically during the second or third decades) and a high incidence of hearing loss and ocular anomalies (6,12,13). Females usually show only urinary abnormalities, but a small percentage can develop ESRD (14,15). In the autosomal recessive form, the clinical and morphological features are identical to those observed in male XLAS patients (16). The autosomal dominant form is milder and rarely associated with extra-renal manifestations, but a significant fraction of patients (29%) progresses to renal failure later in life (mean age of 56 years) (10,17,18). Genotype-phenotype correlations have been established, especially in the more frequent X-linked form where truncating mutations closer to the 5' end are associated with a more severe phenotype (19). However, the wide spectrum of phenotypic variability associated with ATS even within the same family has suggested the possibility of more complex inheritance patterns. Building on this clinical evidence, through a collaborative effort of several European centers and using massive

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4 parallel sequencing, we have recently demonstrated the existence of digenic inheritance
5 in ATS with two mutations in the alpha3-4-5 collagen IV genes (20). Interestingly, this
6 'two-locus model' could explain the variable expressivity of the disease better than
7 simple Mendelian inheritance. Preliminary results suggest that individuals with
8 heterozygous mutations in different genes develop renal failure later than those with X-
9 linked or autosomal recessive Alport syndrome in line with molecule stoichiometry of
10 the disruption of the type IV collagen triple helix.
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15 Here, using a combined approach of next generation sequencing (NGS), RNA
16 studies and clinical re-evaluation, we have unraveled non-Mendelian complex
17 mechanisms of inheritance in a set of Alport families. These results underline the
18 importance of reconsidering the mode of inheritance of Alport syndrome in some
19 families and help to explain genotype-phenotype correlations, providing the basis for
20 accurate genetic counseling, with a correct assessment of recurrence risks and
21 prognostic considerations.
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MATERIALS AND METHODS

Patients and families

Patients were selected by nephrologists and/or clinical geneticists from Italian institutes for ATS genetic diagnosis at the Medical Genetics Unit of Siena (21). For each subject, pedigrees were constructed and clinical data were collected regarding kidney function (haematuria, proteinuria, chronic renal failure or ESRD) and extra-renal manifestations (high tone sensorineural hearing loss and ocular lesions). Detailed data on microscopic examination of kidney biopsies were also collected when available. A sample of peripheral blood in EDTA tubes was collected from probands and all available family members (18 subjects). All subjects analyzed in this study gave written informed consent.

Samples and DNA extraction

Genomic DNA was isolated from EDTA peripheral blood samples using a QIAamp DNA Blood Kit according to the manufacturer's protocol (Qiagen, Hilden, Germany). With patients' informed consent the DNA samples were stored in the institutional Telethon biobank (<http://biobanknetwork.telethon.it/>).

Custom panel design

In order to perform mutational screening of patients presenting with a clinical suspicion of ATS we created a custom panel for *COL4A3*, *COL4A4* and *COL4A5* genes using the "Ion AmpliSeq™ designer" software (www.ampliseq.com). We targeted the coding region and all the flanking introns up to 25 bp. The 3' and 5' UTR were not included in the panel design. The total coverage of the panel for the three genes was 98.33% and it consisted of two different PCR primers pools containing 98 and 96 amplicons for a total of 184 amplicons.

Ion Torrent PGM sequencing

The library preparation was performed using the Ion AmpliSeq™ Library Kit 2.0 (Life Technologies). This kit allowed obtaining a barcoded library of the 184 amplicons,

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4 corresponding to the 151 exons of *COL4A3/COL4A4/COL4A5* genes compatible with
5 the Ion PGM platform, according to the Life Technologies protocol
6 (<http://ioncommunity.lifetechnologies.com/community/login.jspa?referer=http://ioncommunity.lifetechnologies.com/community/protocols-home>). Libraries were purified using
7 Agencourt AMPure XP system and quantified using the Qubit® dsDNA HS Assay Kit
8 reagent (Invitrogen Corporation, Life Technologies, Carlsbad, CA, USA), pooled at an
9 equimolar ratio, annealed to carrier spheres (Ion Sphere™ Particles, Life Technologies)
10 and clonally amplified by emulsion PCR (emPCR) using the Ion OneTouch™ 2 system
11 (Ion PGM™ Template OT2 200 kit, Life Technologies). The spheres, carrying single
12 stranded DNA templates, were loaded to Ion 314™, 316™ or 318™ chip and
13 sequenced on the Ion Torrent PGM, using the Ion PGM™ Sequencing 200 kit v2,
14 according to the protocol of Life Technologies. Post run analysis was conducted using
15 the latest version (v5.0.2) of the data analysis software Torrent Suite™ (Life
16 Technologies). Coverage assessment was performed using the “coverageAnalysis”
17 plug-in (v5.0.2) that gives information about the amplicon read coverage and variants
18 were called using the “variantCaller” plug-in (5.0.2).

31 32 **Sanger sequencing and segregation analysis**

33 Variant validation and segregation analysis in DNA of all available family members
34 were performed by direct Sanger sequencing. Sequencing was performed on an ABI
35 Prism 310 genetic analyzer (PE Applied Biosystems) and data were analyzed by
36 Sequencher software V.4.9 (Gene Codes, Ann Arbor, USA). Genotypes of pedigrees
37 were examined to determine the pattern of inheritance of the variants identified and to
38 assess genotype–phenotype correlations.

44 45 **Variant interpretation**

46 47 ***Bioinformatic analysis***

48 Identified variants were checked in the datasets of the following on-line databases:
49 NCBI dbSNP Build 142 (<http://www.ncbi.nlm.nih.gov/projects/SNP/>), ExAC (Exome
50 Aggregation Consortium; <http://exac.broadinstitute.org/>) and 1000 genomes
51 (<http://www.1000genomes.org>). Our in-house data set containing exome data of 80
52 Italian individuals was also consulted. In order to check whether variants have been
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4 already reported in ATS we consulted literature and the specific *COL4A5* mutation
5 database

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7 (http://www.arup.utah.edu/database/ALPORT/ALPORT_display.php?sort=2#alport;

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9 Last update: October 2013) (22). *In silico* evaluation of variants pathogenicity was
10 performed using Alamut software v2.3 (Interactive Biosoftware, Rouen, France) which
11 includes the following prediction tools: Align GVD, SIFT, MutationTaster, PolyPhen-
12 2, SpliceSiteFinder-like, MaxEntScan, NNSPLICE, GeneSplicer, Human Splicing
13 Finder, and ESE Finder.
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17 18 19 ***Minigene assay for intronic variants***

20 To obtain the hybrid minigene constructs, we employed as backbone the β -globin-
21 pCDNA3.1 vector harboring an artificial multiple cloning site (containing the XhoI,
22 NotI and HindIII sites) as previously reported (23). We amplified by PCR a portion of
23 each gene containing the variant of interest (c.780+5G>A in *COL4A5*, c.931-2A>G and
24 c.976-56A>G in *COL4A4*) from genomic DNA of the patients. Primers carried the XhoI
25 and HindIII restriction sites to enable the subsequent cloning in the β -globin-pCDNA3.1
26 vector (oligonucleotides and PCR conditions are available upon request). For the
27 c.780+5G>A variant in intron 13 of the *COL4A5* gene, we obtained a 586 bp fragment
28 including exon 13 and part of the upstream and downstream introns, whereas in the case
29 of the two variants in the *COL4A4* gene (c.931-2A>G in intron 15 and c.976-56A>G in
30 intron 16) we amplified a 847 bp fragment encompassing both exons 16 and 17 and part
31 of their flanking introns. After digestion, the PCR products were cloned into the β -
32 globin-pCDNA3.1 minigene, cut similarly, and the correctness of the construct was
33 verified by direct sequencing. For each of the three variants, we retained one clone
34 containing the wild-type allele and one with the mutation for the expression analysis.
35 The mutated minigene constructs, their corresponding wild-type versions or the empty
36 vector were transfected in HEK cells and after 24 hours total RNA was extracted using
37 the TRIzol kit (Invitrogen) and retrotranscribed as previously reported (24). The cDNA
38 was then amplified using primers located on β -globin exon 2 and 3 in order to avoid
39 eventual interference from the endogenous gene. PCR fragments were analyzed by
40 electrophoresis on standard agarose gels and individual bands were excised and
41 sequenced using the amplification primers.
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RESULTS

In the present study, using NGS (Ion Torrent PGM platform), we identified four ATS families harboring mutations in two distinct collagen IV genes (digenic model) (Fig. 1) (Table 1). In three patients (proband of families 1, 2 and 4), we found a combination of two mutations in *COL4A3* and *COL4A4* genes, whereas in the remaining one (proband of family 3) we identified an association of a *COL4A5* and a *COL4A3* mutation (Fig. 1) (Table 1). Using Sanger sequencing, mutations were confirmed and segregation analysis was performed in all available family members (Fig. 1) (Table 1). Segregation analysis allowed us to verify which family members presented the same combination of variants in two collagen genes and which presented only one variant in heterozygous/hemizygous state. We also identified a case (family 5) suggestive of digenic inheritance with intronic variants in two collagen IV genes outside the canonical GT-AT splice sites consensus sequences: c.976-56A>G in *COL4A4* and c.780+5G>A in *COL4A5* (Fig. 1) (Table 2). The c.976-56A>G change in *COL4A4* created an alternative 5' splice site according to all the used in silico programs (5/5) (Table 2), while 4 out of 5 programs predicted a weakening of the 5' canonical splice site for the c.780+5G>A variant in *COL4A5* (Fig. 2) (Table 2). These variants were not reported in examined databases (dbSNP NCBI, 1000 genomes, ExAC and our in-house data set).

Among the identified mutations, three had been already previously reported in ATS (Table 2) (25-27). One of them (p.(Gly1277Ser)) in *COL4A3* was found in 3 out of 5 families of our cohort combined with a second mutation in *COL4A4* (family 1 and 2) or *COL4A5* (family 3) (Fig. 1) (Table 1). This missense change (rs190598500) is associated with a minor allele frequency (MAF) of 0.06% in dbSNP142 and 0.03% in ExAC database. Among identified *COL4A4* novel variants, one (p.(Gly1233Arg)) is a missense change involving Glycine residue in the Gly-X-Y triplet repeats of the collagenous domain (a.a. 62-1459) and the other (c.931-2A>G) is a splicing mutation consisting of an intronic substitution in the canonical donor site (Table 2). One novel mutation detected in *COL4A3* (p.(Asn1508Ser)) is a missense change involving a highly conserved residue (Asparagine) in the non-collagenous domain (aa 1440-1671) (Table 2). This variant (rs200512461) is associated with a MAF of 0.02% in ExAC database.

Pathogenicity assessment of splicing variants by minigene assay

In order to explore whether three of the intronic variants, reported in Table 2, could affect splicing, we decided to use a hybrid minigene approach to overcome the limitation of an undetectable expression level of *COL4* transcripts in blood samples. In particular, for the analysis of the effects of the two variants in the *COL4A4* gene, we cloned into a β -globin minigene backbone a single fragment including both exons 16 and 17 and part of the flanking upstream and downstream introns. HEK cells were transfected with either the wild-type or the mutant constructs and expression analysis by RT-PCR showed that the c.931-2A>G variant leads to an aberrant splicing product due to a complete skipping of exon 16 (Fig. 2 A). Although some degree of exon skipping was observed also with the wild-type construct, no wild-type transcript was produced from the mutant minigene, confirming the pathogenicity of the mutation, as predicted by the disruption of the canonical acceptor splice site, which leads to a lack of exon definition by the spliceosomal machinery (28). Conversely, expression of the construct harbouring the c.976-56A>G single substitution produces the same splicing pattern as the wild-type and does not affect splicing neither by means of an exon skipping nor by means of intronic retention, thus behaving like a benign variant (Fig. 2A).

We used the same minigene-based approach to test the effects on splicing of the c.780+5G>A variant of *COL4A5*. Constructs containing the wild type or the mutant exon 13 (carrying the c.780+5G>A) and part of the flanking introns of *COL4A5* were expressed in HEK cells and cDNA was analysed by RT-PCR using vector-specific primers. As shown in Fig. 2B, the wild-type vector produced a single band corresponding to the correctly spliced transcript, whereas the c.780+5G>A mutant construct yielded two bands, one corresponding to the wild-type construct and one resulting from the skipping of exon 13. Although lack of the 93 nt-long exon 13 does not cause a frameshift, this transcript is likely to be not functional, as the deleted region encodes for critical residues of the collagenous domain and, in addition, other adjacent mutations causing skipping of the same exon have been already reported as pathogenic in ATS (29). The co-existence of both the wild-type and the aberrant transcript is a phenomenon frequently observed for splicing mutation which often presents with a “leaky effect” (30,31) and it is in line with the hypothesis of an hypomorphic allele mutation.

Families Description

Digenic autosomal inheritance with mutations on the same homologous chromosome

In two families with *COL4A3*/*COL4A4* mutation combinations (1 and 2), the two mutations were *in cis* configuration, inherited together on the same chromosome (Fig. 1). In these cases, the inheritance pattern mimics an autosomal dominant form with a recurrence risk of 50%. However, the phenotype is more severe respect to an autosomal dominant pattern. Accordingly, in family 1 and 2, subject III:1 and II:2 progressed towards renal insufficiency at 53 and 37 years, respectively. (Fig 1 and Table 1).

Digenic unlinked autosomal/X-linked inheritance

In proband (II:1) of family 3, we detected a *de novo* mutation in *COL4A5* combined with a *COL4A3* mutation inherited from the unaffected father. The female patient presents an atypically severe phenotype in relation to the age (8 years), showing hematuria and proteinuria (Fig. 1 and Table 1). Both her father (II; 44 years) and her sister (II:2; 7 years) carrying only one mutation in *COL4A3* are asymptomatic (Fig. 1 and Table 1).

Digenic autosomal inheritance with mutations on different homologous chromosomes

In family 4, the two mutations in *COL4A3* and *COL4A4* were inherited independently, likely indicating an *in trans* configuration. This kind of transmission mimics the autosomal recessive inheritance with a 25% probability of having another child with the same genotype. However, the severity of the phenotype is intermediate between an autosomal dominant and an autosomal recessive form. Indeed, the proband (II:1), carrying the two heterozygous mutations, presented CRF at 40 years and ESRD at 49 years. His brother of 47 years (II:2), carrying only one mutation in *COL4A4*, shows microhematuria and proteinuria with a normal renal function (Fig. 1 and Table 1).

Monogenic X-linked inheritance due to a hypomorphic splicing mutation

In family 5, we hypothesized a digenic mode of transmission involving a combination of intronic mutations in *COL4A4* and *COL4A5* on the basis of bioinformatic splicing tools (Fig. 2). However, the minigene assay demonstrated that the variant in *COL4A4* does not affect splicing and that the *COL4A5* variant represents a “leaky” splicing mutation. It is therefore a case of X-linked inheritance caused by a hypomorphic allele. The proband (IV:1) of 8 years with both variants presented isolated hematuria. Both the father (III:1) with the benign variant in *COL4A4* and the mother (III:2) with the hypomorphic variant in *COL4A5* are healthy (Fig. 1 and Table 1). In the maternal branch, we observed 3 affected male subjects, two of them presenting hypoacusis and ESRD at the age of 33 and 48 years (II:2 and II:3). The other male subject (III:5), aged 23 years, has hematuria, proteinuria and bilateral keratoconus. On the maternal side, we also observed two female patients, one died at 76 years with ESRD starting at 70 years of age (I:1) and one of 52 years with referred hypoacusis (II:5).

DISCUSSION

In 2015, through a collaborative international effort of several European centers, we reported the occurrence of digenic inheritance in Alport syndrome (ATS) (20). In the present study, by using an NGS panel for simultaneous analysis of *COL4A3*, *COL4A4* and *COL4A5* genes, we provide further evidence that Alport syndrome can be transmitted with an inheritance pattern that goes beyond the Mendelian model, which can explain the age of onset and clinical presentations. The identified digenic cases included: three patients with a combination of mutations in *COL4A3* and *COL4A4* genes (family 1, 2 and 4) and one patient with a combination of variants in *COL4A3* and *COL4A5* genes (family 3). While the first combination has already been reported, the last observation is described in the present study for the first time (20). This evidence confirms that all the combinations of mutated collagen IV genes are possible in accordance with the equal interaction of the three α molecules ($\alpha3$, $\alpha4$ and $\alpha5$) in the formation of the triple helix and suggests that the variable clinical expressivity is likely to be related to the amount of residual functional activity of the non-mutated *COL4* chains.

Among the collagen IV mutations identified in digenic families, three had already been reported in ATS (25-27). In particular, the p.((Gly1277Ser)) mutation in *COL4A3* has been previously described in heterozygous state in a patient with the autosomal dominant form and here was found in three unrelated ATS families, combined with *COL4A4* (family 1 and 2) or *COL4A5* mutations (family 3) (25). Interestingly, two of these families (family 1 and 2) come from the same geographic area in Italy (Campania), suggesting a possible founder effect. Among the identified novel mutations, one in *COL4A4* (p.(Gly1233Arg)) is a “typical” Glycine substitution in the collagenous domain, where this residue is crucial for the correct coiling of the triple helix in the GBM structure (Table 2) (1). Another novel mutation in *COL4A4* (c.931-2A>G) is a splicing change in the canonical acceptor site, leading to a complete skipping of exon 16 as confirmed by minigene assay (Fig. 2A) (Table 2). The novel mutation in *COL4A3* is a missense change (p.(Asn1508Ser)) involving a highly conserved amino acid in the non-collagenous domain of the protein that is fundamental for both triple-helix assembly and collagen IV protomers network formation (1,34).

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4 This study, presenting four new ATS families with digenic inheritance,
5 contributes to characterize the associated phenotype and reinforces the importance for
6 clinicians taking into consideration the possibility of more complex models of
7 transmission in order to assess the correct recurrence risk and define the prognosis.
8 Overall, in accordance with the previous work, ATS patients with mutations in two
9 different collagen genes show a more severe phenotype compared with those with a
10 single mutation (20) (Fig. 1) (Table 1). The mean age of onset of ESRD in double
11 heterozygous patients (proband III:1, family 1; II:2, family 2; II:1, family 4) is 45
12 years, in line with the previously reported data (20). This provides further evidence that
13 renal failure in double heterozygotes occurs at an age intermediate between the
14 autosomal dominant and the recessive form, suggesting a *COL4*-mutated chain dose-
15 dependent effect. Therefore while in cases with mutations on the same autosomal
16 chromosome (*in cis*) (Families 1 and 2), the risk assessment would not have changed
17 in comparison with a monogenic autosomal dominant inheritance, notably, a clinical re-
18 evaluation on the basis of molecular data highlights the importance of considering a
19 worse prognosis (10,20,33,35). Conversely, a better prognosis should be considered in
20 comparison with an autosomal recessive mode of inheritance, if the two mutations are
21 independently inherited on chromosome 2 (*in trans*) (Family 4) (11,20,36). In sporadic
22 cases (Family 3) it is important to consider the possibility of an underlying inherited
23 mutation, whose effect, being cumulative with a *de novo* mutation can determine a
24 worse clinical progression.

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39 This study underlines the importance of firmly establishing the pathogenicity of
40 the identified variants in cases of suspected multi-allelic inheritance. In fact, while NGS
41 technology has the advantage of simultaneously detecting all the variants present in
42 gene-panels, it also raises major problems concerning variant interpretation. In this
43 study, NGS identified one patient with two potentially pathogenic splicing variants
44 (c.976-56A>G and c.780+5G>A) on the basis of in silico prediction tools (Table 2).
45 However, minigene approach revealed that only one of the two variants affects splicing
46 (c.780+5G>A) and also demonstrated that the variant has a “leaky” effect leading to a
47 hypofunctional *COL4A5* protein. Interestingly, these findings are in accordance with an
48 X-linked “milder” presentation of affected subjects. On the maternal side, one male
49 patient (II:3) developed ESRD at the age of 48 years, an older age than expected in a
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4 classic X-linked form (Fig. 1, Table 1). In cases of intronic variants outside the
5 canonical splice site, it is particularly important to accompany NGS screening with
6 RNA studies or minigene assay if expression tissues are not easily accessible in order to
7 avoid an overestimation of cases with more complex inheritance and erroneous
8 identification of at risk members.
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12 In conclusion, this study provides further evidence of digenic inheritance in ATS
13 and underlines the importance of considering a non-Mendelian transmission in the
14 clinical evaluation. Indeed this mode of inheritance can explain the variable clinical
15 expression of the disease in comparison to Mendelian models. The identification of
16 cases with atypical inheritance by NGS is fundamental since it leads to reconsider the
17 recurrence risk in the families and opens new perspectives in the management of genetic
18 and prenatal counseling for ATS.
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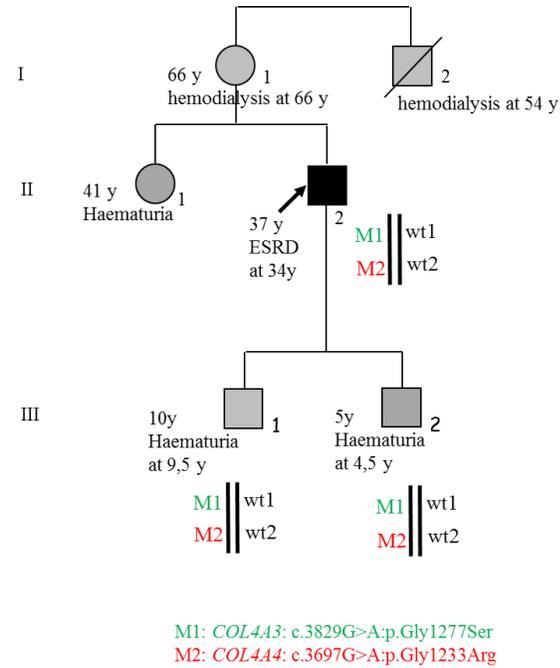
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Fig. 1. Pedigree of the five families presenting variants in two different collagen IV genes indicating possible digenic inheritance. Panels A/B: Pedigrees of families 1 (A) and 2 (B) where probands show mutations in *COL4A3* and *COL4A4* on the same homologous chromosome (*in cis* configuration). The p.(Gly1277Ser) variant is in common to the two families while the *COL4A4* variants are: a splicing change in family 1 (c.931-2A>G) and a Glycine substitution in family 2 (p.(Gly1233Arg)). **Panel C:** Pedigree of family 3 where the proband presents the p.(Gly1277Ser) variant in *COL4A3* inherited from the father and a de novo variant in *COL4A5* (p.(Gly869Arg)). **Panel D:** Pedigree of family 4 where the proband has mutations in *COL4A3* (p.(Asn1508Ser)) and *COL4A4* (p.(Arg1682Gln)) likely inherited independently (*in trans* configuration). **Panel E:** Pedigree of family 5 where the proband shows intronic variants in *COL4A4* (c.976-56A>G) and *COL4A5* (c.780+5G>A) with a possible effect on splicing. Probands are indicated by arrows. Squares indicate males; circles, females; black symbols, individuals with clinical symptoms ranging from proteinuria to renal failure; grey symbols, subjects with isolated haematuria; crossed symbols, deceased individuals. ESRD, end-stage renal disease; CRF, chronic renal failure; CKD, chronic kidney disease, eGFR, estimated glomerular filtration rate.

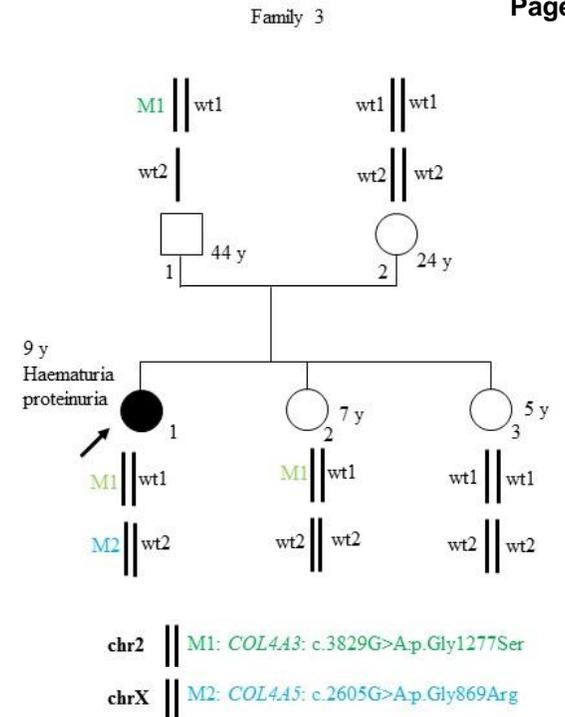
Fig. 2 RT-PCR analysis of the *COL4A4* (Fig.2A) and *COL4A5* (Fig.2B) minigene constructs expressed in HEK cells using vector specific primers. A schematic representation of the hybrid minigene constructs is depicted on the left; the dotted lines indicate normal splicing. **Fig.2A** *COL4A4* exons 16-17, construct containing exons 16 and 17 and part of flanking introns of *COL4A4* gene. Legend: M, 1kb plus molecular marker (Invitrogen); Empty, empty β -globin-pCDNA3.1 vector; WT, wild type construct; c.976-56A>G and c.931-2A>G constructs containing the c.976-56A>G or c.931-2A>G variants, respectively. **Fig. 2B:** *COL4A5* exon 13 is the construct containing exon 13 and part of upstream and downstream introns of *COL4A5* gene. Legend as above; c.780+5G>A, construct containing the c.780+5G>A variant.

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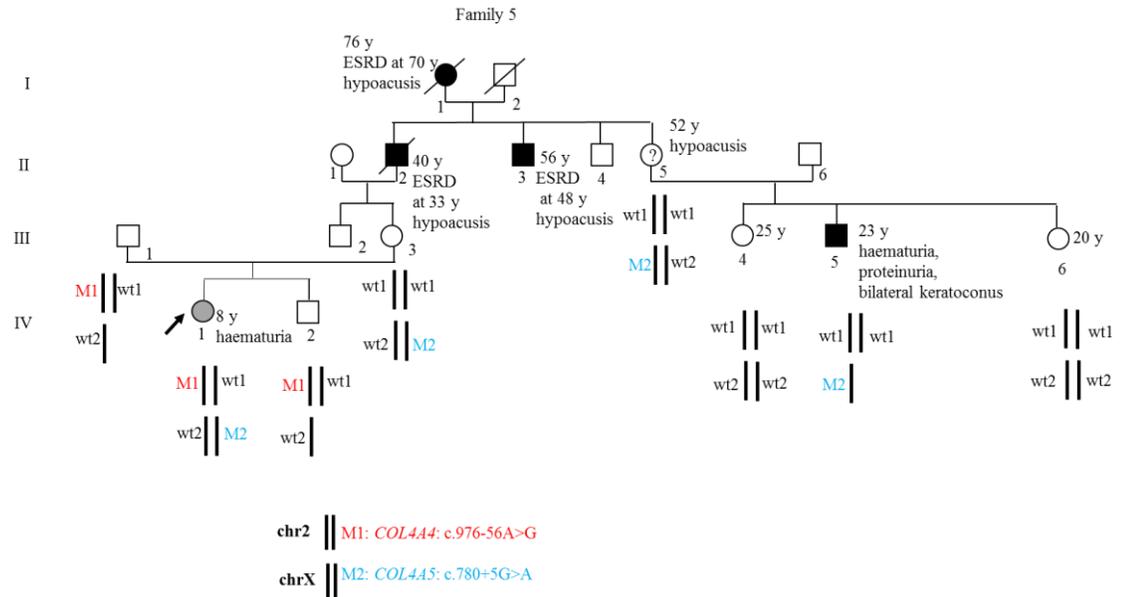
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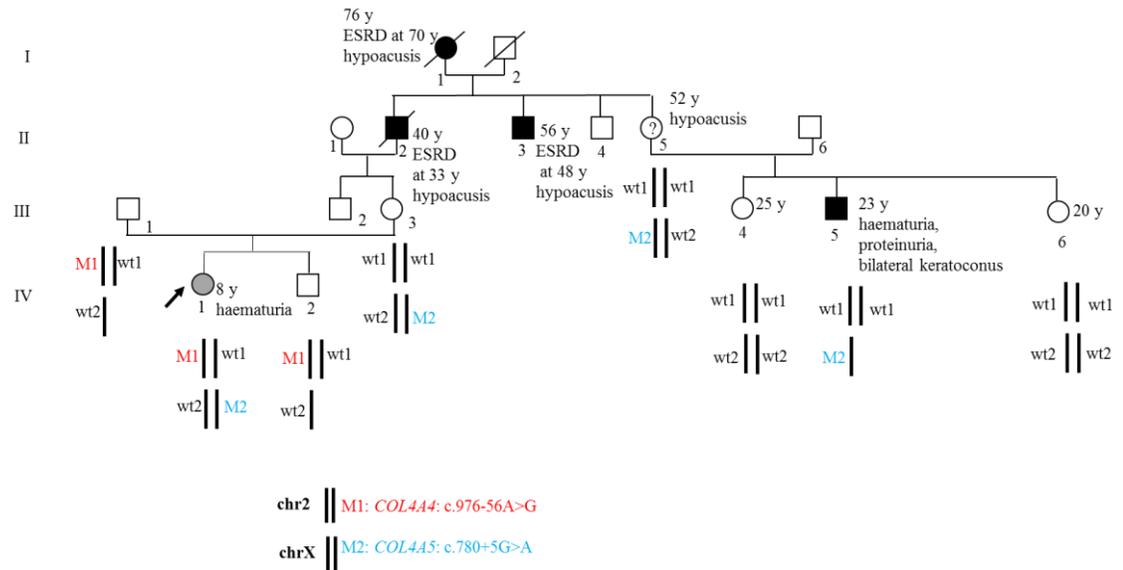
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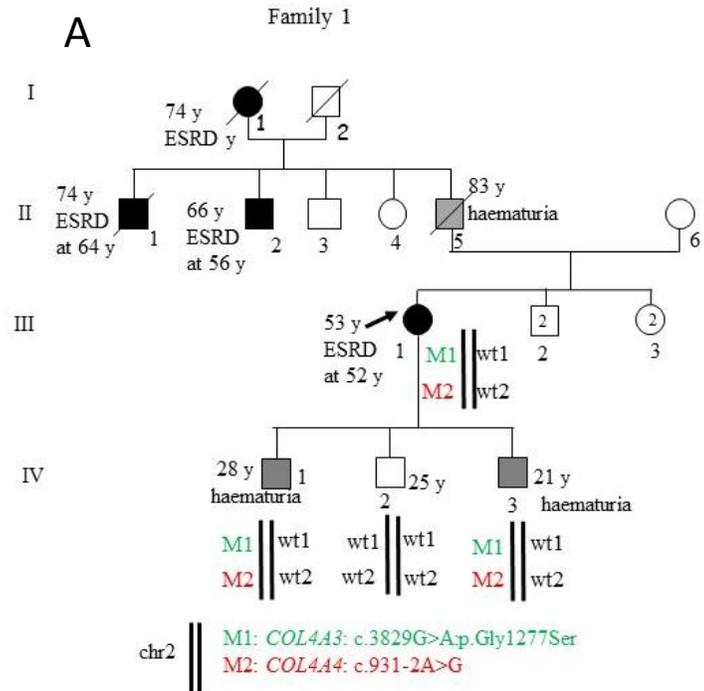
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Family 5



A



Family 4

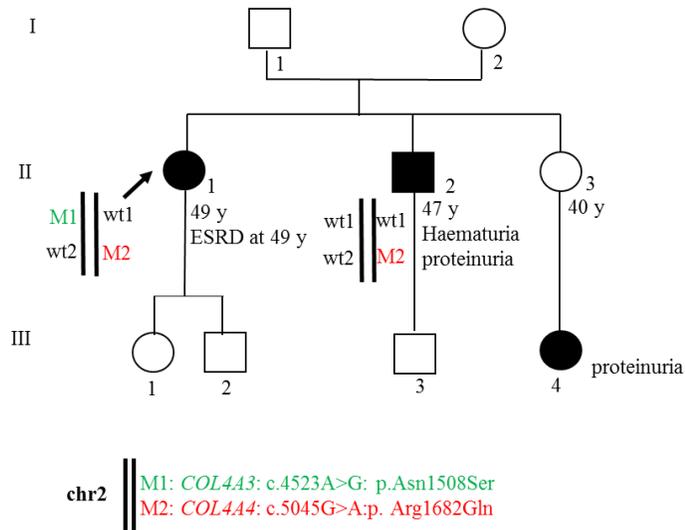


Fig. 2a COL4A4 exons 16-17

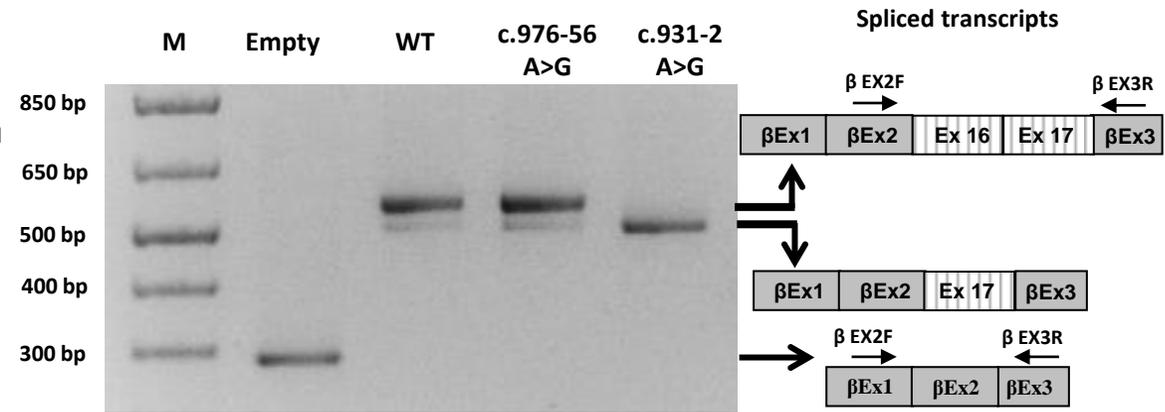
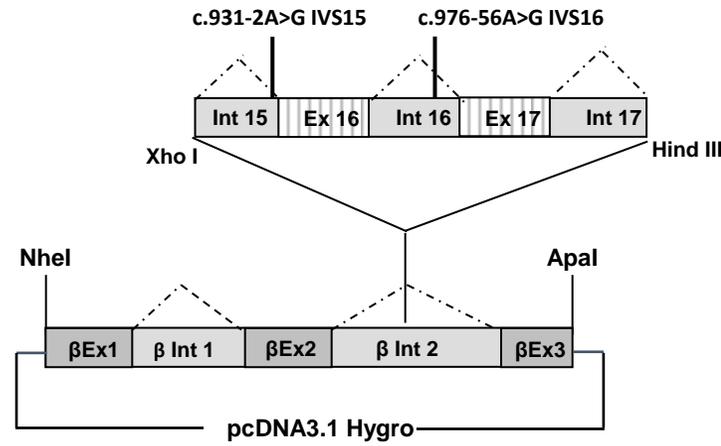


Fig. 2b COL4A5 exons 13

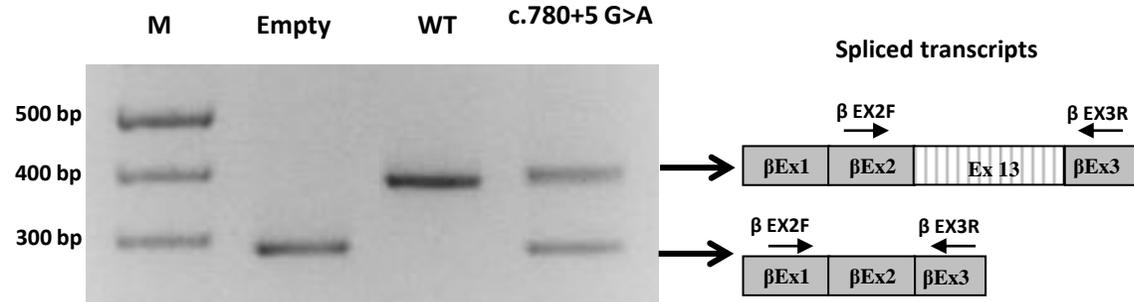
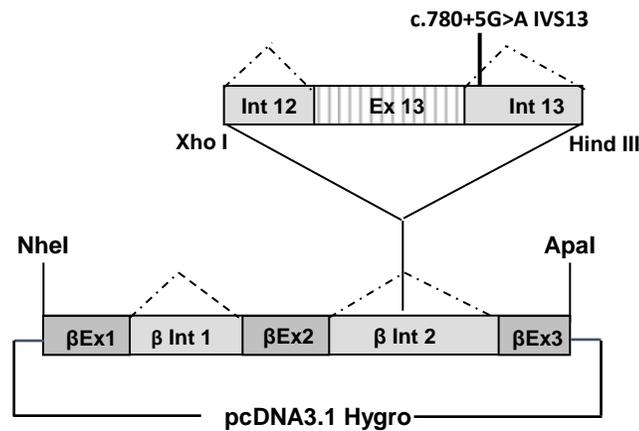


Table 1. Clinical characteristics and collagen gene variants in 5 probands with Alport syndrome and their family members.

Family number	Family member (pedigree position)	Sex	Age, years	Kidney disease (age, years)	Renal biopsy	Hearing loss (age, years)	Ocular lesions	Mutation (nucleotide change; effect protein)		
								<i>COL4A3</i>	<i>COL4A4</i>	<i>COL4A5</i>
1	Proband (III:1)	F	53	microhaematuria, proteinuria, CRF (52)	Yes (thinning, splitting and thickening, basket-weaving and mesangial humps)	no	no	c.3829G>A; p.(Gly1277Ser)	c.931-2A>G	None
1	Paternal grandmother (I:1)	F	dead at 74	ESRD	NA	NA	NA	NT	NT	NT
1	Father (II:5)	M	dead at 83	microhaematuria	NA	NA	NA	NT	NT	NT
1	Uncle (II:1)	M	74	ESRD at 64	NA	NA	NA	NT	NT	NT
1	Uncle (II:2)	M	66	ESRD at 56	NA	NA	NA	NT	NT	NT
1	Son (IV:1)	M	28	microhaematuria (21)	NA	no	no	c.3829G>A; p.(Gly1277Ser)	c.931-2A>G	NT
1	Son (IV:2)	M	25	no	NA	no	no	Variant not present	Variant not present	NT
1	Son (IV:3)	M	21	micro/macrohaematuria (19)	NA	no	no	c.3829G>A; p.(Gly1277Ser)	c.931-2A>G	NT
2	Proband (II:2)	M	37	microhaematuria, proteinuria, ESRD (34)	NA	no	no	c.3829G>A; p.(Gly1277Ser)	c.3697G>A; p.(Gly1233Ser)	None
2	Mother (I:1)	F	66	CRF	NA	yes (47)	NA	NT	NT	NT
2	Uncle (I:2)	M	dead	ESRD	NA	NA	NA	NT	NT	NT
2	Sister (II:1)	F	41	microhaematuria (34 Y)	NA	NA	NA	NT	NT	NT
2	Son (III:1)	M	10	microhaematuria	NA	NA	NA	c.3829G>A; p.(Gly1277Ser)	c.3697G>A; p.(Gly1233Ser)	NT
2	Son (III:2)	M	5	microhaematuria	NA	NA	NA	c.3829G>A; p.(Gly1277Ser)	c.3697G>A; p.(Gly1233Ser)	NT
3	Proband (II:1)	F	9	micro/macrohaematuria, persistent proteinuria (8)	Yes (thinning and splitting)	no	no	c.3829G>A; p.(Gly1277Ser)	None	c.2605G>A; p.(Gly869Arg)
3	Father (I:1)	M	43	no	NA	NA	NA	c.3829G>A; p.(Gly1277Ser)	NT	Variant not present
3	Mother (I:2)	F	34	no	NA	NA	NA	Variant not present	NT	Variant not present

3	Sister (II:2)	F	5	no	NA	NA	NA	c.3829G>A; p.(Gly1277Ser)	NT	Variant not present
3	Sister (II:3)	F	4	no	NA	NA	NA	Variant not present	NT	Variant not present
4	Proband (II:1)	F	49	microhaematuria, proteinuria, ESRD (49)	Yes (thinning)	no	no	c.4523A>G; p.(Asn1508Ser)	c.5045G>A; p.(Arg1682Gln)	None
4	Brother (II:2)	M	47	microhaematuria, proteinuria	NA	NA	NA	Variant not present	c.5045G>A; p.(Arg1682Gln)	NT
4	Sister (II:3)	F	40	no	NA	NA	NA	NT	NT	NT
4	Nephew (III:4)	F	?	proteinuria	NA	NA	NA	NT	NT	NT
5	Proband (IV:1)	F	8	microhaematuria	Yes (thinning, splitting and thickening and mesangial humps)	no	no	None	c.976-56A>G	c.780+5G>A
5	Brother (IV:2)	M	6	no	NA	no	no	NT	c.976-56A>G	Variant not present
5	Great grandmother (I:1)	F	dead at 76	ESRD	NA	yes	NA	NT	NT	NT
5	Grandfather (II:2)	M	dead at 40	ESRD	NA	yes	NA	NT	NT	NT
5	Paternally relatedr (II:3)	M	56	ESRD	NA	yes	NA	NT	NT	NT
5	Paternally related(II:5)	F	52	NA	NA	yes	NA	NT	Variant not present	c.780+5G>A
5	Maternally related (III:2)	F	17	ESRD	NA	NA	NA	NT	NT	NT
5	Father (III:1)	M	35	no	NA	no	no	NT	c.976-56A>G	Variant not present
5	Mother (III:3)	F	28	no	NA	NA	NA	NT	Variant not present	c.780+5G>A
5	Maternally related(III:4)	F	25	no	NA	NA	NA	NT	Variant not present	Variant not present
5	Maternally related(III:5)	M	23	haematuria/persistent proteinuria	NA	no	bilateral cherato conus	NT	Variant not present	c.780+5G>A
5	Maternally related (III:6)	F	20	no	NA	NA	NA	NT	Variant not present	Variant not present

CRF, chronic renal failure; ESRD, end-stage renal disease; NA, data missing or DNA not available for analysis; NT, gene not tested in relatives because not mutated in proband. Mutations were all named in accordance with the standard nomenclature guidelines proposed by the Human Genome Variation Society (<http://www.hgvs.org>). Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence (*COL4A5*, RefSeq NM_000495.4; *COL4A4*, RefSeq NM_000092; *COL4A3*, RefSeq NM_000091).

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Table 2. Molecular features and predicted pathogenicity of 5 variants in collagen IV genes, found in 3 patients with Alport syndrome.

Variant	Type	Exon/ Intron	dbSNP ID	Effect on protein	Protein domain	Missense pathogenicity*		Splicing Effect*						Reference
						SIFT (score) ^a	Mutation taster (p value) ^b	PolyPhen2 (score) ^c	SpliceSite Finder	MaxEntScan	NNSPLICE	GeneSplicer	Human Splicing Finder	
COL4A3														
c.3829G>A	Missense	43	rs190598500 ^d	p(Gly1277Ser)	Collagenous	D(0)	DC(1)	PD (1)	-	-	-	-	-	<i>Heidet L et al. J Am Soc Nephrol. 2001 Jan;12(1):97-106</i>
c.4523A>G	Missense	49	rs200512461 ^e	p(Asn1508Ser)	NC1	D(0)	DC(1)	PD (1)	-	-	-	-	-	none
COL4A4														
c.931-2A>G	Misplicing Loss of a 3' canonical AG splice site	Intron 15	-	-	Collagenous	-	-	-	SL	SL	ND	ND	SL	none
c.976-56A>G	Not effect splicing	Intron 16	-	-	Collagenous	-	-	-	AS	AS	AS	AS	AS	none
c.3697G>A	Missense	39	-	p.(Gly1233Arg)	Collagenous	D(0)	DC(1)	PD (1)	-	-	-	-	-	none
c.5045G>A	Missense	48	rs36844711 ^f	p.(Arg1682Gln)	NC1	D(0)	DC(1)	PD (1)	-	-	-	-	-	<i>Rana K. et al. Pediatr Nephrol 2007 May; 22(5):652-7</i>
COL4A5														
c.2605G>A	Missense	31	rs104886189 ^g	p.(Gly869Arg)	Collagenous	D(0)	DC(1)	PD (1)	-	-	-	-	-	<i>Strasser K. et al. Nephrol Dial Transplant. 2012 Nov;27(11):4236-40</i>
c.780+5G>A	Misplicing hypomorphic allele	Intron 13	-	-	Collagenous	-	-	-	WS (95.6;83.5) _h	WS (10.7→6.6) ^h	ND (1→1) ^h	WS (5.9→2.4) ^h	WS (98.8→86.7) ^h	none

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4 *Pathogenicity predicted using Alamut software v.2.3 (Interactive Biosoftware, Rouen, France), indicating functional impact of variants with relevant prediction tools. For
5 missense variants, data derived from SIFT, MutationTaster, and PolyPhen-2 are detailed. Splicing predictions included MaxEntScan, NNSPLICE, Human Splicing Finder,
6 SpliceSiteFinder, GeneSplicer predictions tools. ^a Substitutions with normalized probabilities <0.05 are predicted to be deleterious, those ≥ 0.05 are predicted to be tolerated. ^b P
7 value indicates the security of the prediction as either 'disease-causing' or 'polymorphism', with 1 being most secure. ^c Benign, possibly damaging, and probably damaging
8 correspond to posterior probability intervals [0, 0.2], (0.2, 0.85), and [0.85, 1], respectively. ^d Minor allele frequency/count=0.0006/3; ^e dbSNP clinical significance not available,
9 ExAC minor allele frequency =0,022%; ESP minor allele frequency=0,08%; ^f dbSNP clinical significance not available, ExAC minor allele frequency =0,0075%; ESP minor
10 allele frequency=0,02%; ^gdbSNP clinical significance, pathogenic; ^hsplicing prediction score: score value of wildtype sequence → score value of mutated sequence. NA, not
11 applicable. D, deleterious. DC, disease causing. PD, probably damaging. SL, loss of canonical splice site. AS, add splice site. WS, weakening of canonical splice site. ND Not
12 determined by the tool. NCI, main non collagenous domain. CRF, chronic renal failure; ESRD, end-stage renal disease; NA, data missing or DNA not available for analysis; NT,
13 gene not tested in relatives because not mutated in proband.

14 Mutations were all named in accordance with the standard nomenclature guidelines proposed by the Human Genome Variation Society (<http://www.hgvs.org>). Nucleotide
15 numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence (*COL4A5*, RefSeq NM_000495.4;
16 *COL4A4*, RefSeq NM_000092; *COL4A3*, RefSeq NM_000091).
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