



Alport syndrome: impact of digenic inheritance in patients management

This is a pre print version of the following article:												
Original:												
Fallerini, C., Baldassarri, M., Trevisson, E., Morbidoni, V., La Manna, A., Lazzarin, R., et al. (2017). Alport syndrome: impact of digenic inheritance in patients management. CLINICAL GENETICS, 92(1), 34-44 [10.1111/cge.12919].												
Availability:												
This version is availablehttp://hdl.handle.net/11365/1002134 since 2017-12-07T16:03:32Z												
Published:												
DOI:10.1111/cge.12919												
Terms of use:												
Open Access The terms and conditions for the reuse of this version of the manuscript are specified in the publishing policy. Works made available under a Creative Commons license can be used according to the terms and conditions of said license. For all terms of use and more information see the publisher's website.												

(Article begins on next page)

The impact of non-Mendelian inheritance in Alport syndrome

Chiara Fallerini¹, Margherita Baldassarri^{1,2}, Eva Trevisson^{3,4}, Valeria Morbidoni^{3,4}, Angela La Manna⁵, Roberta Lazzarin⁶, Andrea Pasini⁷, Giancarlo Barbano⁸, Angela Rosa Pinciaroli⁹, Guido Garosi¹⁰, Elisa Frullanti¹, Anna Maria Pinto^{1,2}, Maria Antonietta Mencarelli², Francesca Mari^{1,2}, Alessandra Renieri^{1,2}, Francesca Ariani^{1,2}.

1-Medical Genetics, University of Siena, Siena, Italy

2-Genetica Medica, Azienda Ospedaliera Universitaria Senese, Siena, Italy

3-Department of Woman and Child Health, University of Padova, 35128 Padova, Italy

4- Istituto di Ricerca Pediatria, IRP, Città della Speranza, 35129 Padova, Italy

5-Department of Pediatrics, Second University of Napoli, Napoli, Italy

6-Nephrology and Dialysis, Ospedale San Giacomo Apostolo, Castelfranco Veneto, Italy

7-Nephrology and Pediatric Dialysis, Ospedale S. Orsola Malpighi, Bologna, Italy 8- Renal immunopathology, Istituto Giannina Gaslini, Genova, Italy.

9- Nephrology, Dialysis, Azienda Ospedaliera Pugliese Ciaccio, Catanzaro, Italy 10-Nephrology, Dialysis and Transplantation, Azienda Ospedaliera Universitaria Senese, Siena, Italy

Corresponding author:

Alessandra Renieri M.D., Ph.D. Full Professor of Medical Genetics University of Siena Director of Medical Genetics Unit Azienda Ospedaliera Universitaria Senese Viale Bracci 2 53100 Siena, Italy Phone: 39 0577 233303 FAX 39 0577 233325 E.mail: alessandra.renieri@unisi.it

Conflict of interest: no conflict of interest to declare.

Acknowledgments: we would like first to thank ATS patients and their families. We are also grateful to Professor Francies Flinter (Department of Clinical Genetics, Guy's Hospital, London, SE1 9RT, UK) and Doctor Helen Storey (Molecular Genetics Laboratory, Viapath, Guy's Hospital, London, SE1 9RT) for critical review of the manuscript. We would like to thank the Cell lines and DNA bank of Rett Syndrome, X-linked mental retardation and other genetic diseases and the Cell line and DNA Bank of Genetic Movement Disorders and Mitochondial Diseases (GMD-MDbank), member of the Telethon Network of Genetic Biobanks (project no. GTB12001), funded by Telethon Italy, and the EuroBioBank network. This work was supported by Italian Ministry of Health Grant GR-2009-1578914 and University of Padova Grant CPDA140508/14 (to E.T.).

Abstract

Alport syndrome (ATS) is a genetically heterogeneous nephropathy with considerable phenotypic variability and different transmission patterns, including Mendelian (Xlinked/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 (Xg22.3), 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

parallel sequencing, we have recently demonstrated the existence of digenic inheritance in ATS with two mutations in the alpha3-4-5 collagen IV genes (20). Interestingly, this 'two-locus model' could explain the variable expressivity of the disease better than simple Mendelian inheritance. Preliminary results suggest that individuals with heterozygous mutations in different genes develop renal failure later than those with Xlinked or autosomal recessive Alport syndrome in line with molecule stoichiometry of the disruption of the type IV collagen triple helix.

Here, using a combined approach of next generation sequencing (NGS), RNA studies and clinical re-evaluation, we have unraveled non-Mendelian complex mechanisms of inheritance in a set of Alport families. These results underline the importance of reconsidering the mode of inheritance of Alport syndrome in some families and help to explain genotype-phenotype correlations, providing the basis for accurate genetic counseling, with a correct assessment of recurrence risks and prognostic considerations.

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 (<u>http://biobanknetwork.telethon.it/</u>).

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 AmpliSeqTM 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,

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

Sanger sequencing and segregation analysis

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

Variant interpretation

Bioinformatic analysis

Identified variants were checked in the datasets of the following on-line databases: NCBI dbSNP Build 142 (<u>http://www.ncbi.nlm.nih.gov/projects/SNP/</u>), ExAC (Exome Aggregation Consortium; <u>http://exac.broadinstitute.org/</u>) and 1000 genomes (<u>http://www.1000genomes.org</u>). Our in-house data set containing exome data of 80 Italian individuals was also consulted. In order to check whether variants have been

Clinical Genetics

already reported in ATS we consulted literature and the specific COL4A5 mutation database

(http://www.arup.utah.edu/database/ALPORT/ALPORT_display.php?sort=2#alport; Last update: October 2013) (22). *In silico* evaluation of variants pathogenicity was performed using Alamut software v2.3 (Interactive Biosoftware, Rouen, France) which includes the following prediction tools: Align GVGD, SIFT, MutationTaster, PolyPhen-2, SpliceSiteFinder-like, MaxEntScan, NNSPLICE, GeneSplicer, Human Splicing Finder, and ESE Finder.

Minigene assay for intronic variants

To obtain the hybrid minigene constructs, we employed as backbone the β -globinpCDNA3.1 vector harboring an artificial multiple cloning site (containing the XhoI, NotI and HindIII sites) as previously reported (23). We amplified by PCR a portion of each gene containing the variant of interest (c.780+5G>A in COL4A5, c.931-2A>G and c.976-56A>G in COL4A4) from genomic DNA of the patients. Primers carried the XhoI and HindIII restriction sites to enable the subsequent cloning in the β -globin-pCDNA3.1 vector (oligonucleotides and PCR conditions are available upon request). For the c.780+5G>A variant in intron 13 of the COL4A5 gene, we obtained a 586 bp fragment including exon 13 and part of the upstream and downstream introns, whereas in the case of the two variants in the COL4A4 gene (c.931-2A>G in intron 15 and c.976-56A>G in intron 16) we amplified a 847 bp fragment encompassing both exons 16 and 17 and part of their flanking introns. After digestion, the PCR products were cloned into the β globin-pCDNA3.1 minigene, cut similarly, and the correctness of the construct was verified by direct sequencing. For each of the three variants, we retained one clone containing the wild-type allele and one with the mutation for the expression analysis. The mutated minigene constructs, their corresponding wild-type versions or the empty vector were transfected in HEK cells and after 24 hours total RNA was extracted using the TRIzol kit (Invitrogen) and retrotranscribed as previously reported (24). The cDNA was then amplified using primers located on β -globin exon 2 and 3 in order to avoid eventual interference from the endogenous gene. PCR fragments were analyzed by electrophoresis on standard agarose gels and individual bands were excised and sequenced using the amplification primers.

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 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 (I1; 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 bionformatic 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 (α 3, α 4 and α 5) in the formation of the triple helix and suggests that the variable clinical espressivity 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).

Clinical Genetics

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

This study underlines the importance of firmly establishing the pathogenicity of the identified variants in cases of suspected multi-allelic inheritance. In fact, while NGS technology has the advantage of simultaneously detecting all the variants present in gene-panels, it also raises major problems concerning variant interpretation. In this study, NGS identified one patient with two potentially pathogenic splicing variants (c.976-56A>G and c.780+5G>A) on the basis of in silico prediction tools (Table 2). However, minigene approach revealed that only one of the two variants affects splicing (c.780+5G>A) and also demonstrated that the variant has a "leaky" effect leading to a hypofunctional *COL4A5* protein. Interestingly, these findings are in accordance with an X-linked "milder" presentation of affected subjects. On the maternal side, one male patient (II:3) developed ESRD at the age of 48 years, an older age than expected in a

classic X-linked form (Fig. 1, Table 1). In cases of intronic variants outside the canonical splice site, it is particularly important to accompany NGS screening with RNA studies or minigene assay if expression tissues are not easily accessible in order to avoid an overestimation of cases with more complex inheritance and erroneous identification of at risk members.

In conclusion, this study provides further evidence of digenic inheritance in ATS and underlines the importance of considering a non-Mendelian transmission in the clinical evaluation. Indeed this mode of inheritance can explain the variable clinical expression of the disease in comparison to Mendelian models. The identification of cases with atypical inheritance by NGS is fundamental since it leads to reconsider the recurrence risk in the families and opens new perspectives in the management of genetic and prenatal counseling for ATS.

REFERENCES

1. Gubler MC. Inherited diseases of the glomerular basement membrane. *Nat Clin Pract Nephrol* 2008; **1**:24-37.

2. Hudson BG, Tryggvason K, Sundaramoorthy M, Neilson EG. Alport's syndrome, Goodpasture's syndrome, and type IV collagen. *N Engl J Med* 2003; **348**:2543–56.

3. Alport AC. 1927. Hereditary familial congenital haemorrhagic nephritis. Br Med Jr 19;1(3454):504-6.

4. Kashtan CE. Alport syndrome. An inherited disorder of renal, ocular, and cochlear basement membranes. *Medicine (Baltimore)* 1999; **78(5):**338-60.

5. Feingold J, Bois E, Chompret A, Broyer M, Gubler MC, Grünfeld JP, *et al.* Genetic heterogeneity of Alport syndrome. *Kidney Int* 1985; **27**:672–7.

6. Barker DF, Hostikka SL, Zhou J, Chow LT, Oliphant AR, Gerken SC, *et al.* Identification of mutations in the COL4A5 collagen gene in Alport syndrome. Science 1990; 8;248(4960):1224-7.

7. Lemmink HH, Mochizuki T, van den Heuvel LP, Schröder CH, Barrientos A, Monnens LA, *et al.* Mutations in the type IV collagen alpha 3 (COL4A3) gene in autosomal recessive Alport syndrome. *Hum Mol Genet* 1994; **3**:1269–73.

8. Mochizuki T, Lemmink HH, Mariyama M, Antignac C, Gubler MC, Pirson Y, *et al.* Identification of mutations in the alpha 3(IV) and alpha 4(IV) collagen genes in autosomal recessive Alport syndrome. *Nat Genet* 1994; **8**:77–81.

9. Longo I, Porcedda P, Mari F, Giachino D, Meloni I, Deplano C, *et al.* COL4A3/COL4A4 mutations: from familial hematuria to autosomal-dominant or recessive Alport syndrome. **Kidney Int** 2002; 61:1947–56.

10. Fallerini C, Dosa L, Tita R, Del Prete D, Feriozzi S, Gai G, *et al.* Unbiased next generation sequencing analysis confirms the existence of autosomal dominant Alport syndrome in a relevant fraction of cases. *Clin Genet* 2014; **86**:252–7.

11. Wang Y, Sivakumar V, Mohammad M, Colville D, Storey H, Flinter F, *et al.* Clinical and genetic features in autosomal recessive and X-linked Alport syndrome. *Pediatr Nephrol* 2014; **29**:391–6.

12. Renieri A, Bruttini M, Galli L, Zanelli P, Neri T, Rossetti S, *et al.*. X-linked Alport syndrome: an SSCP-based mutation survey over all 51 exons of the COL4A5 gene. *Am J Hum Genet* 1996; **58(6):**1192-204.

13. Jais JP, Knebelmann B, Giatras I, De Marchi M, Rizzoni G, Renieri A, *et al.* X-linked Alport syndrome: natural history in 195 families and genotype- phenotype correlations in males. *J Am Soc Nephrol* 2000; **11(4)**:649-57.

14. Gross O, Netzer KO, Lambrecht R, Seibold S, Weber M. Meta-analysis of genotype-phenotype correlation in X-linked Alport syndrome: impact on clinical counselling. Nephrol Dial Transplant 2002; 17(7):1218-27.

15. Jais JP, Knebelmann B, Giatras I, De Marchi M, Rizzoni G, Renieri A, et al. Xlinked Alport syndrome: natural history and genotype-phenotype correlations in girls and women belonging to 195 families: a "European Community Alport Syndrome Concerted Action" study. J Am Soc Nephrol 2003; 14(10):2603-10.

16. Gubler MC, Knebelmann B, Beziau A, Broyer M, Pirson Y, Haddoum F, *et al.* Autosomal recessive Alport syndrome: immunohistochemical study of type IV collagen chain distribution. *Kidney Int* 1995. **47(4)**:1142-7.

17. Marcocci E, Uliana V, Bruttini M, Artuso R, Silengo MC, *et al.*. Autosomal dominant Alport syndrome: molecular analysis of the COL4A4 gene and clinical outcome. *Nephrol Dial Transplant* 2009; **24**:1464–71.

18. Hoefele J, Lange-Sperandio B, Ruessmann D, Glöckner-Pagel J, Alberer M, Benz MR, *et al.* Novel heterozygous COL4A3 mutation in a family with late-onset ESRD. Pediatr Nephrol 2010; **25(8)**:1539-42.

19. Bekheirnia MR, Reed B, Gregory MC, McFann K, Shamshirsaz AA, Masoumi A, *et al.* Genotype-phenotype correlation in X-linked Alport syndrome. *J Am Soc Nephrol* 2010; **21(5)**:876-83.

20. Mencarelli MA, Heidet L, Storey H, van Geel M, Knebelmann B, Fallerini C, *et al.* Evidence of digenic inheritance in Alport syndrome. Med Genet 2015; **52**:163–174.

21. Flinter FA, Cameron JS, Chantler C, Houston I, Bobrow M. Genetics of classic Alport's syndrome. *Lancet*. 1988; **29;2(8618)**:1005-7.

22. Crockett DK, Pont-Kingdon G, Gedge F, Sumner K, Seamons R, Lyon E. The Alport syndrome COL4A5 variant database. *Hum Mutat* 2010; **31**:E1652-7.

23. Forzan M, Salviati L, Pertegato V, Casarin A, Bruson A, Trevisson E, *et al.* Is CFTR 621+3 A>G a cystic fibrosis causing mutation?. *J Hum Genet* 2010; 55(1):23-6.

24. Trevisson E, Salviati L, Baldoin MC, Toldo I, Casarin A, Sacconi S, Cesaro L, Basso G, Burlina AB. (2007) Argininosuccinate lyase deficiency: mutational spectrum

in Italian patients and identification of a novel ASL pseudogene. Hum Mutat. 28:694-702.

25.Heidet L, Arrondel C, Forestier L, Cohen-Solal L, Mollet G, Gutierrez B, *et al.* Structure of the human type IV collagen gene COL4A3 and mutations in autosomal Alport syndrome. *J Am Soc Nephrol* 2001; **12(1)**:97-106.

26. Rana K, Tonna S, Wang YY, Sin L, Lin T, Shaw E, *et al.* Nine novel COL4A3 and COL4A4 mutations and polymorphisms identified in inherited membrane diseases. *Pediatr Nephrol* 2007; **22(5)**:652-7.

27. Strasser K, Hoefele J, Bergmann C, Büscher AK, Büscher R, Hoyer PF, *et al.* COL4A5-associated X-linked Alport syndrome in a female patient with early inner ear deafness due to a mutation in MYH9. *Nephrol Dial Transplant* 2012; **27(11)**:4236-40.

28. Cartegni L, Chew SL, Krainer AR. Listening to silence and understanding nonsense: exonic mutations that affect splicing. *Nat Rev Genet* 2002; **3**:285-98.

29. Tazón-Vega B, Ars E, Burset M, Santín S, Ruíz P, Fernández-Llama P, *et al.* Genetic testing for X-linked Alport syndrome by direct sequencing of COL4A5 cDNA from hair root RNA samples. *Am J Kidney Dis.* 2007; **50(2)**:257.e1-14.

30. Raben N, Nichols RC, Martiniuk F, Plotz PH. A model of mRNA splicing in adult lysosomal storage disease (glycogenosis type II). *Hum Mol Genet* 1996; **5**:995-1000.

31. Vezain M, Gérard B, Drunat S, Funalot B, Fehrenbach S, N'Guyen-Viet V, *et al.* A leaky splicing mutation affecting SMN1 exon 7 inclusion explains an unexpected mild case of spinal muscular atrophy. Hum Mutat 2011; **32(9)**:989-94.

32. Jefferson JA, Lemmink HH, Hughes AE, Hill CM, Smeets HJ, Doherty CC, et al. Autosomal dominant Alport syndrome linked to the type IV collagene alpha 3 and alpha 4 genes (COL4A3 and COL4A4). *Nephrol Dial Transplant* 1997; **Aug;12(8)**:1595-9.

33. Pescucci C, Mari F, Longo I, Vogiatzi P, Caselli R, Scala E, *et al.*. Autosomaldominant Alport syndrome: natural history of a disease due to COL4A3 or COL4A4 gene. *Kidney Int* 2004; **65(5)**:1598-603.

34. Boutaud A, Borza DB, Bondar O, Gunwar S, Netzer KO, Singh N, *et al.* Type IV collagen of the glomerular basement membrane. Evidence that the chain specificity of network assembly is encoded by the noncollagenous NC1 domains. *J Biol Chem* 2000; **275**: 30716-30724.

35. Artuso R, Fallerini C, Dosa L, Scionti F, Clementi M, Garosi G, *et al.* Advances in Alport syndrome diagnosis using next-generation sequencing. *Eur J Hum Genet* 2012; **20(1)**:50-7.

36. Zhang Y, Wang F, Ding J, Zhang H, Zhao D, Yu L, *et al.* Genotype-phenotype correlations in 17 Chinese patients with autosomal recessive Alport syndrome. Am J Med Genet A. 2012; **158A(9)**:2188-93.

Titles and legends to figure:

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)) COL4A4 (p.(Arg1682Gln)) likely inherited independently (in and 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.



chr2 M1. COL4A3: c.4525A>G. p.Ash1508Ser M2: COL4A4: c.5045G>A:p. Arg1682Gh

41

42

Clinical Genetics

hemodialysis at 66 y

37 y

ESRD

at 34y

hemodialysis at 54 y

M1 wt1

M2 wt2

5y

M1: COL4A3: c.3829G>A:p.Gly1277Ser

M2: COL4A4: c.3697G>A:p.Gly1233Arg

Haematuria

M1 wt1

M2 wt2

at 4,5 y

66 y

В

T

Π

III

Ε

41 y

Haematuria

10y

at 9,5 y

Haematuria

M1 wt1

M2 wt2





chr2 M1: COL4A4: c.976-56A>G chrX M2: COL4A5: c.780+5G>A

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)			
	(1.1.8) (1.1.1.)					(COL4A3	COL4A4	COL4A5	
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)	М	dead at 83	michroematuria	NA	NA	NA	NT	NT	NT	
1	Uncle (II:1)	М	74	ESRD at 64	NA	NA	NA	NT	NT	NT	
1	Uncle (II:2)	М	66	ESRD at 56	NA	NA	NA	NT	NT	NT	
1	Son (IV:1)	М	28	microhaematuria (21)	NA	no	no	c.3829G>A; p.(Gly1277Ser)	c.931-2A>G	NT	
1	Son (IV:2)	М	25	no	NA	no	no	Variant not present	Variant not present	NT	
1	Son (IV:3)	М	21	micro/macrohaemathuria (19)	NA	no	no	c.3829G>A; p.(Gly1277Ser)	c.931-2A>G	NT	
2	Proband (II:2)	М	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)	М	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)	М	10	microhaematuria	NA	NA	NA	c.3829G>A; p.(Gly1277Ser)	c.3697G>A; p.(Glv1233Ser)	NT	
2	Son (III:2)	М	5	microhaematuria	NA	NA	NA	c.3829G>A; p.(Gly1277Ser)	c.3697G>A; p.(Gly1233Ser)	NT	
3	Proband (II:1)	F	9	micro/macrohaemathuria, persistent proteinuria (8)	Yes (thinning and splitting)	no	no	c.3829G>A; p.(Gly1277Ser)	None	c.2605G>A; p.(Gly869Arg)	
3	Father (I:1)	М	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	

Clinical Genetics

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)	М	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)	М	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)	М	dead at 40	ESRD	NA	yes	NA	NT	NT	NT
5	Paternally relatedr (II:3)	М	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)	М	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)	М	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).

Clinical Genetics

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

Variant		Exon/ Intron	dbSNP ID	Effect on protein	Protein domain	Missense pathogenicity*			Splicing Effect*					
	Туре					SIFT (score) ^a	Mutation taster (p value) ^b	PolyPhen2 (score) ^c	SpliceSite Finder	MaxEntScan	NNSPLICE	GeneSplicer	Human Splicing Finder	— Reference
COL4A3						•1 •			•1			•		
c.3829G>A	Missense	43	rs190598500 ^d	p.(Gly1277Ser)	Collagenous	D(0)	DC(1)	PD (1)	-	-	-	-	-	Heidet L et al. J Am Soc Nephrol. 2001 Jan;12(1):97-106
c.4523A>G	Missense	49	rs200512461 ^e	p.(Asn1508Ser)	NC1	D(0)	DC(1)	PD (1)	-	-	-	-	-	none
c.931-2A>G	Misplicing Loss of a 3' canonical AG splice site	Intron 15	-	-	Collagenous	-	8	4	SL	SL	ND	ND	SL	none
c.976-56A>G	Not effect	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)	-	-	-	-	-	Rana K. et al. Pediatr Nephrol 2007 May; 22(5):652-7
COL4A5														Sturger og V. et el
c.2605G>A	Missense	31	rs104886189 ^g	p.(Gly869Arg)	Collagenous	D(0)	DC(1)	PD (1)	-	-	-	-	-	Nephrol Dial Transplant. 2012 Nov:27(11):4236-40
c.780+5G>A	Misplicing hypomorphic allele	Intron 13	-	-	Collagenous	-	-	-	WS (95.6;83.5 h	$ \overset{\text{WS}}{(10.7 \rightarrow 6.6)} $	$^{h} ND (1 \rightarrow 1)$	WS) ^h (5.9→2.4	$WS \\ (98.8 \rightarrow 86.7)^{h}$	none

12 13 15 16 17 18 19 $\begin{array}{c} 20\\ 21\\ 22\\ 23\\ 24\\ 25\\ 26\\ 27\\ 28\\ 29\\ 30\\ 31\\ 32\\ 33\\ 35\\ 36\\ 37\\ 38\\ 39\\ 40\\ 41 \end{array}$ 43 44 47 48

*Pathogenicity predicted using Alamut software v.2.3 (Interactive Biosoftware, Rouen, France), indicating functional impact of variants with relevant prediction tools. For missense variants, data derived from SIFT, MutationTaster, and PolyPhen-2 are detailed. Splicing predictions included MaxEntScan, NNSPLICE, Human Splicing Finder, 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 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 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, ExAC minor allele frequency =0,022%; ESP minor allele frequency=0,08%; ^f dbSNP clinical significance not available, ExAC minor allele frequency=0,02%; ^gdbSNP clinical significance, pathogenic; ^hsplicing prediction score: score value of wildtype sequence→ score value of mutated sequence. NA, not 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 determined by the tool. NC1, main non collagenous domain. 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).