Phenotype variability in a large Spanish family with Alport syndrome associated with novel mutations in COL4A3 gene

C. Cervera-Acedo 1, A. Coloma 2, E. Huarte-Loza 2, M. Sierra-Carpio 2 and E. Domínguez-Garrido 1*

Abstract

Background: Alport syndrome is an inherited renal disorder characterized by glomerular basement membrane lesions with hematuria, proteinuria and frequent hearing defects and ocular abnormalities. The disease is associated with mutations in genes encoding α3, α4, or α5 chains of type IV collagen, namely COL4A3 and COL4A4 in chromosome 2 and COL4A5 in chromosome X. In contrast to the well-known X-linked and autosomal recessive phenotypes, there is very little information about the autosomal dominant. In view of the wide spectrum of phenotypes, an exact diagnosis is sometimes difficult to achieve.

Methods: We investigated a Spanish family with variable phenotype of autosomal dominant Alport syndrome using clinical, histological, and genetic analysis.

Results: Mutational analysis of COL4A3 and COL4A4 genes showed a novel heterozygous mutation (c. 998G > A; p.G333E) in exon 18 of the COL4A3 gene. Among relatives carrying the novel mutation, the clinical phenotype was variable. Two additional COL4A3 mutations were found, a Pro-Leu substitution in exon 48 (p.P1461L) and a Ser-Cys substitution in exon 49 (p.S1492C), non-pathogenics alone.

Conclusion: Carriers of p.G333E and p.P1461L or p.S1492C mutations in COL4A3 gene appear to be more severely affected than carriers of only p.G333E mutation, and the clinical findings has an earlier onset. In this way, we could speculate on a synergistic effect of compound heterozygosity that could explain the different phenotype observed in this family.

Keywords: Alport syndrome, COL4A3 gene, Microhematuria, Proteinuria, genotype-phenotype correlation

Background

Alport syndrome (AS) is an inherited renal disorder characterized by glomerular hematuria, proteinuria, progressive renal failure, often associated with bilateral sensorineural hearing loss, and ocular abnormalities [1]. AS accounts for 0.6–2% of patients who start renal replacement therapy in Europe, with a frequency of about 1 in 5000 people, although that number is probably overestimated [2, 3]. The disease is genetically heterogeneous and associated with mutations in genes encoding α3, α4, or α5 chains of type IV collagen that forms a distinct network in the glomerular basement membrane (GBM) [4, 5]. Mutations in COL4A5 gene located in the Xq22 and encoding the α5 (IV) chain, are responsible for the X-linked form of AS (XLAS, OMIM 301050) with an estimated frequency of 80–85% [6]. This type of AS is more severe in men than in women. Before the age of 30 years (juvenile form), 70% of affected males reach end-stage renal disease (ESRD), whereas the remaining 30% progress toward ESRD after their 30 years (rare adult form). Females with XLAS usually have only microhematuria, however, there are some cases in which they are as affected as males [7–10]. Dominant and recessive autosomal forms of AS (ADAS, OMIM 104200 and ARAS, OMIM 203780) are characterized by mutations in COL4A3 and COL4A4 genes, located in 2q36.3, encoding the α3 and α4 chains of IV collagen [11, 12]. The frequency estimated for ARAS and ADAS are 15%
and 1–5%, respectively [13]. ARAS is associated with homozygous or compound heterozygous mutations in either COL4A3 or COL4A4 genes. The disease is equally severe in males and females, and early progression toward ESRD is frequent before the end of the second decade of life [14–16]. Clinical and morphologic features are comparable to the XLAS [17]. In ADAS, both female and male patients are affected in the same way and this type shows high clinical variability, from hematuria to late onset ESRD, associated in few cases with hearing loss [15–19].

Heterozygous mutations in COL4A4 or COL4A3 genes have been found also in patients with benign familial hematuria (BFH, OMIM 141200), that is clinically defined by persistent glomerular hematuria and by the absence of extra-renal findings [20, 21]. The diagnosis of AS is straightforward when clinical renal and extra-renal symptoms as well as family history are typical of this disease. However, the lack of family history does not exclude the diagnosis of AS, as approximately 10% of COL4A5 mutations occur de novo [17]. In the presence of sporadic persistent hematuria associated with proteinuria without any other symptoms suggesting AS, renal biopsy is frequently the first investigation performed. Examination of the renal tissue can exclude other hematric glomerular disease, being the most frequent IgA nephropathy, and often shows typical ultrastructural abnormalities of the GBM when electron microscopy (EM) is performed [17].

The identification of the mode of inheritance of AS is essential for providing genetic counselling to affected families. Clinical and family history together with morphologic phenotypes do not always allow to conclude the type of transmission of the disease. The analysis of the type IV collagen chains expression in skin and/or kidney basement membranes can make it easier [22]. However, the expression of these collagen chains is normal in about one third of the families, and it does not exclude the diagnosis at all. In these cases, molecular testing might be required to make a definitive diagnosis [23]. The identification of new mutations and their correlation with clinical phenotype are important to offer an early treatment and familiar genetic counselling.

Here, we report the analysis of the COL4A3 and COL4A4 genes in a Spanish family with variable clinical phenotype of ADAS.

**Methods**

This study was conducted according to protocols approved by the Ethical Committee of La Rioja (CEICLAR PI-161), and written informed consent was obtained from each patient for publication. The proband and 18 members of the family (11 males and 8 females), with clinical suspect of AS, were included in this study. Familiar clinical history was performed carefully in each case by reconstruction of the pedigree for at least three generations (Fig. 1). Urinalysis and renal function evaluations were carried out in all patients. Audiological and ophthalmic examinations were performed, and, whenever indicated, a renal biopsy was analysed by LM and the ultrastructural study was performed by EM.

Proband (V.I) is a 31-year-old male patient that was referred to the Pediatric Nephrology Service at the age of 8 years for evaluation of persistent hematuria. He fulfilled three of the AS clinical diagnostic criteria: a positive family history of hematuria, proteinuria and hearing loss. Ophthalmic evaluation was normal. The results of audiometric testing revealed a bilateral sensorineural hearing loss. At the age of 13 years, urine analysis revealed proteinuria (1.77 g/24 h), so he underwent a renal biopsy. LM revealed minimal mesangial changes, with no glomeruli or tubulointerstitial lesions, and the immunofluorescence microscopy was negative for IgA, IgG, IgM, C3 and C4. He started treatment with inhibitors of angiotensin converting enzyme (ACE inhibitors). Nevertheless, progressive increase in proteinuria levels (up to 5.2 g/24 h) with clinical and biochemical nephrotic syndrome were found. For this reason, a second renal biopsy was performed. The results were compatible with thin basement membrane disease (TBMD). Antiproteinuric treatment was reinforced with ACE inhibitors and spironolactone. Due to the persistence of nephrotic syndrome, tacrolimus treatment was started, but no response after 10 months of treatment was obtained. At the age of 25 years, he started with progressive renal failure and three years later he received renal transplantation from living related donor. Considering the clinical course of the patient, and keeping in mind the hearing impairment and the family history, molecular genetic testing was carried out. This study was extended to all of family members (10 males and 8 females, age ranges from 25 to 66 years). In order to determine the molecular cause of the disease, blood samples from the proband and his family were collected after genetic counselling and informed consent. Genomic DNA and RNA were isolated from EDTA peripheral blood samples using QIAamp DNA Blood kit and RNeasy Mini kit, respectively, according to the manufacturer’s protocol (Qiagen, http://www.qiagen.com). Mutation analysis of COL4A3 (NM_000091) and COL4A4 (NM_000092) genes was performed by analyzing the entire coding sequences and flanking intronic regions by PCR and subsequent Sanger sequencing in both directions. PCR products were purified with ExoStar™ (GE Healthcare). Sequencing reactions were performed using Big Dye® Terminator v.3.1 Cycle Sequencing kit (Applied Biosystems, Forest City, CA, USA), and purified with Big Dye X-Terminator (Applied Biosystems, Foster City, CA, USA). DNA sequences were analyzed on an ABI PRISM® 3130
Fig. 1 Pedigree of the family where mutations of COL4A3 gene were found. Squares are males and circles are females. An oblique bar indicates a deceased individual. The arrow indicates the proband patient.
automated sequencer (PE Applied Biosystems, Forest City, CA, USA) with Sequencing Analysis Software (Applied Biosystems, Foster City, CA). After that, the obtained sequences were analyzed using SeqScape v2.5 software. The specific primer sequences are available upon request. Pathogenicity of the detected variants was predicted by in silico analysis with bioinformatics tools such as Sorting Intolerant From Tolerant (SIFT), Mutation Taster, Polyphen-2, and Human Splicing Finder (HSF 3.0). ExAC browser of Broad Institute, 1000 Genomes database and dbSNP138.

Results
Renal morphological findings
In the first biopsy from the proband (V.1), LM revealed minimal abnormalities in the mesangial area with normal glomeruli, tubules and interstitium. In the second one, LM showed lesions with areas of tubular atrophy, focal interstitial fibrosis and a slight lymphoid infiltrate, without changes in the GBM or vascular damage. Masson’s trichrome staining revealed glomeruli with segmental hyalinization. Thinning, characteristically pronounced thickening of the GBM and weakened podocyte adhesion to the GBM were found by EM (Fig. 2). These findings were compatible with the diagnosis of TBMD.

Genetic analysis
Analysis of all exons of the COL4A3 and COL4A4 genes revealed the presence of a novel heterozygous mutation c. 998G > A in exon 18 resulting in p.G333E of the COL4A3 gene. No mutations were found in COL4A4 gene. p.G333E mutation was found in the proband and it was confirmed in 12 members of the family (9 males and 3 females). The mutation was absent in 100 healthy controls. We used PolyPhen and SIFT to predict possible functional effects, and the variant was classified as possibly damaging. No further pathogenic COL4A3 mutations were detected in this family. A Pro-Leu heterozygous substitution in exon 48 (c.4382C > T; p.P1461L) was found in the proband (V.1), his mother (IV.2) and his sister (V.2), and a Ser-Cys heterozygous substitution in exon 49 (c.4474A > T; p.S1492C) was found in two proband’s cousins (V.3 and V.4) and in his aunt in law (IV.3), both in COL4A3 gene, with an unclear pathogenicity. These mutations have not been described before and were absent in 100 healthy controls.

Genotype-phenotype correlation
Detailed clinical information of the 19 patients belonging to this family has been collected in Table 1. Regarding the clinical manifestations associated with the p.G333E mutation, hematuria was found in 100% of cases, while hearing loss and proteinuria were presented in 6/13 (46.1%) and 8/13 (61.5%) patients, respectively. Nephrotic-range proteinuria was observed in 4/13 (30.7%) of the patients, 3 males and 1 female. 75% of patients with proteinuria in the nephrotic range showed renal failure, and it accounts for 23% of the carriers. Renal biopsy was obtained from 1 of these males (the proband), and the results were compatible with the diagnosis of focal segmental glomerulosclerosis (FSGS). Ocular lesions were not identified in any case. With respect of the patients with compound heterozygous COL4A3 mutations, p.G333E and p.P1461L (V.1) or p.G333E and p.S1492C (V.3 and V.4), the phenotype was more severe, with an early upset of the disease, and reaching nephrotic syndrome. In addition, V.1 and V.3 patients received a renal transplant from their mothers. On the other hand, the patients carrying only p.P1461L or p.S1492C mutations (IV.2 and IV.4), did not present any clinical manifestations.

Discussion
ADAS is a genetically and phenotypically heterogeneous disease. XLAS is, by far, the commonest, although ADAS
and ARAS have also been described and are said to be the mode of inheritance in 15% of families [24]. Mutations in \textit{COL4A3}, \textit{COL4A4}, or \textit{COL4A5} genes result in a spectrum of phenotypes from BFH to AS [11], including XLAS, ARAS, or ADAS. Accurate genetic counseling and reasonable estimation of prognosis in patients and families with persistent hematuria require precise diagnosis. Comprehensive clinical evaluation and pedigree analysis (including testing of relatives for hematuria) combined with histopathology and study of type IV collagen expression in skin and kidney base membrane are sometimes sufficient to firmly establish a diagnosis and determine the risk of transmission. However, in the majority of cases, molecular analysis is necessary to achieve the desired level of diagnostic accuracy. Mutations in \textit{COL4A3} and \textit{COL4A4} genes have been found to be involved in the pathogenesis of ARAS and ADAS, as well as of TBMD, with a high clinical variability [11, 25]. Most \textit{COL4A3} and \textit{COL4A4} mutations have been detected to cause ARAS, and only a small number of mutations in those genes have been described for TBMD or ADAS [26]. They are also associated with a wide spectrum of phenotypes ranging from isolated microhaematuria to ESRD, as highlighted also by the intra- and inter-clinical variability reported in ADAS. In this form, male to male transmission can be observed, the renal disease progresses in slow way in males as well as in females and, when documented, the EM shows thick, thick or diffuse thin GBM. The hearing loss, when present, is a late event [17]. The clinical features of ADAS are similar to those of X-linked disease. However, deterioration of renal function tends to occur more slowly [12, 27, 28].

In this family, pedigree analysis suggested a dominant inheritance pattern. All exons and their intronic boundaries of \textit{COL4A3} and \textit{COL4A4} genes were analyzed and a novel pathogenic heterozygous mutation in \textit{COL4A3} gene (c. 998G > A; p.G333E) was found. All affected patients carried this mutation. The course of the nephropathy was more severe, with earlier onset in the proband and his cousin compared to their fathers. We found that they carried an additional \textit{COL4A3} mutation on the allele inherited from his mother. The Pro-Leu substitution in exon 48 and the Ser-Cys substitution in exon 49 on one of the maternal chromosomes are not pathogenic in itself as it is reflected in the phenotype of the patients, but may have a secondary effect on the mutated \textit{COL4A3} chain. As the carriers of p.G333E and p.P1461L or p.S1492C mutations appear to be more severely affected than the carrier of only p.G333E mutation, we could speculate on a synergistic effect of compound heterozygosity. However, the possibility of such an effect has been reported in the literature previously [16]. For example, in the case of dystrophic epidermolysis bullosa, a neutral glycine substitution in the \textit{COL7A1} gene increased the effect of a dominant glycine substitution in the other copy of the \textit{COL7A1} gene, resulting to a more severe phenotype in the compound heterozygote [18].

| Table 1: Clinical and molecular data of reported patients |
|--------------------------------------------------------|
| Patient | Gender | Age | Hematuria | Proteinuria | Renal Function | Hearing loss | Ocular lesions | COL4A3 Mutations |
|---------|--------|-----|-----------|-------------|---------------|--------------|--------------|-----------------|
| IV.1    | M      | 58  | Yes       | 1.08 g/24 h | Normal        | Yes          | No           | c.998G > A; p.G333E |
| IV.2    | F      | 56  | No        | No          | Normal        | No           | No           | c.C4382T; p.P1461L |
| IV.3    | M      | 62  | No        | No          | Normal        | No data      | No data      | c.998G > A; p.G333E |
| IV.4    | F      | 66  | No        | No          | Normal        | No data      | No data      | c.A4474T; p.S1492C |
| IV.6    | F      | 57  | No        | No          | Normal        | Yes          | No data      | N.D. |
| IV.7    | M      | 54  | Yes       | 1.26 g/24 h | Normal        | Yes          | No           | c.998G > A; p.G333E |
| IV.9    | M      | 47  | No        | No          | Normal        | No data      | No data      | c.998G > A; p.G333E |
| IV.12   | F      | 50  | No        | No          | Normal        | No           | No           | N.D. |
| IV.19   | F      | 56  | Yes       | >3.5 g/24 h | CRF-dialysis  | Yes          | No           | c.998G > A; p.G333E |
| IV.21   | F      | 53  | Yes       | 0.9 g/24 h  | Normal        | Yes          | No           | c.998G > A; p.G333E |
| V.1     | M      | 31  | Yes       | >3.5 g/24 h | CRF-renal transplant | Yes | No | c.998G > A; p.G333E + c.C4382T; p.P1461L |
| V.2     | F      | 27  | No        | No          | Normal        | No           | No           | c.998G > A; p.G333E |
| V.3     | M      | 34  | Yes       | >3.5 g/24 h | CRF-renal transplant | Yes | No | c.998G > A; p.G333E + c.A4474T; p.S1492C |
| V.4     | M      | 32  | Yes       | >3.5 g/24 h | Nephrotic Syndrome | Yes | No | c.998G > A; p.G333E + c.A4474T; p.S1492C |
| V.5     | F      | 25  | No        | No          | Normal        | No data      | No data      | N.D. |
| V.10    | M      | 27  | Yes       | No          | Normal        | No           | No           | c.998G > A; p.G333E |
| V.11    | M      | 34  | No        | No          | Normal        | No           | No           | N.D. |
| V.14    | M      | 25  | Yes       | No          | Normal        | No data      | No data      | c.998G > A; p.G333E |
| V.16    | M      | 26  | Yes       | 0.35 g/24 h | Normal        | Yes          | No data      | c.998G > A; p.G333E |

CRF: Chronic renal failure, F: female, M: male, N.D.: Not detected.
This family demonstrates that one mutation alone cannot explain the highly variable renal phenotype. We might suppose that the effect of a pathogenic mutation could be influenced by the presence of certain variants in the same genes and/or in other genes that are key players in renal filtration [12]. This is the case of a family with XLAS, in which the coinheritance of COL4A5 mutations and homozygous MYO1E variants were associated with more severe kidney disease [29], and can explain a highly variable renal phenotype, inexplicable by conventional pedigree analysis. As described in this paper, the screening for MYO1E or other non-COL4 podocyte gene mutations in XLAS is suggested when clinical nephropathy is more severe than expected. The dominant nature of COL4A3 mutations and the different clinical manifestations can be explained in several ways. The integration of the altered collagen chain in the final network, thereby disturbing normal structure and functioning [23]. The synthesis of a theoretically equal number of normal and abnormal chains would result in dysfunction [23]. The demonstration of a theoretically equal number of normal and abnormal chains would result in dysfunction [23].

The main clinical manifestation of ADAS is hematuria followed by proteinuria and progressive renal failure [12, 29]. Proteinuria is variable and can be in the nephrotic range, but rarely resulting in nephrotic syndrome. In our family, we observed microscopic hematuria in 100% of p.G333E carrier patients, and 75% of patients with proteinuria in the nephrotic range developed clinical and biochemical nephrotic syndrome.

In contrast with the other forms of AS, loss of renal function is gradual [12, 30]. In our study, the renal failure progression was variable; 70% of p.G333E carriers developed ESRD before the age of 30, while the remaining patients developed it later. We also observed that proteinuria levels greater than 3.5 g/24 h was associated with worst evolution, and unresponsive to treatment with ACE inhibitors, angiotensin II receptor blockers (ARB) [31, 32] or calcineurin inhibitors, such as Tacrolimus [33].

Regarding histological findings, biopsy from the proband revealed areas of thinning of the GBM so a diagnosis of TBMD was done. Thinning of the GBM is non-specific and can be appeared both in BFH and AS. TBMD is recognized as the leading cause of microhematuria and is regarded as a benign condition [34]. Characteristic AS hearing loss occurs in 70% of males before 40 years and in 45% of females with XLAS [8]. Hearing loss is never congenital and it has been always described associated with renal disorder. Its real incidence could be underestimated by lack of audiological examination. For ADAS, hearing loss is not very common, and is around 13 and 27%, based on the studies published [12, 30]. In our family, we observed hearing loss in 46.1% of patients carrying p.G333E mutation.

Typical AS ocular lesions are not present in patients with ADAS in contrast to XLAS and ARAS [30]. These results were also confirmed in our family.

The increasing use of global and unbiased genetic analysis with next-generation sequencing is likely to reveal disease-modifying variants across the range of human genetic disease [29]. The detection of a mutation in affected family members is also important for prenatal diagnosis and subsequent diagnostics of their offspring and could therefore reduce the need for more invasive renal biopsies [35]. The role of molecular diagnosis combined with the mode of transmission may be important for the assessment of the prognosis of the disease, for the differentiation of patients with either TBMD or AS, and may represent a future diagnostic approach [36].

Conclusion

It is very difficult to predict the prognosis in a patient with a heterozygous mutation in either the COL4A3 or the COL4A4 genes. In our study, carriers of p.G333E and p.P1461L or p.S1492C mutations in COL4A3 gene appear to be more severely affected than carriers of only p.G333E mutation, and the clinical findings has an earlier onset. In this way, we could speculate on a synergistic effect of compound heterozygosity that could explain the different phenotype observed in this family. A correct diagnosis and prognosis should be based on a combination of a comprehensive clinical investigation of all family members, including examination of renal and extra-renal signs of AS, associated with a broadly formal molecular genetic analysis of the pedigree.

Abbreviations

ACE: Inhibitors, angiotensin converting enzyme; ADAS: Dominant autosomal form of AS; ARAS: Recessive autosomal form of AS; ARB: Angiotensin II receptor blockers; AS: Alport syndrome; BFH: Benign familial hematuria; EM: Electron microscopy; ESRD: End-stage renal disease; FSGS: Focal segmental glomerulosclerosis; GBM: Glomerular basement membrane; LM: Light microscopy; SIFT: Sorting Intolerant From Tolerant; TBMD: Thin basement membrane disease; XLAS: X-linked form of AS

Acknowledgements

The authors express their gratitude to all patients who participated in this article. We acknowledge Prof Francisco Javier Pardo Mindan from the Department of Pathological Anatomy of University of Navarra Clinic, for electron microscopy analysis. We also thank Dr. Paula Santibáñez, Dr. MaríLópez, and Dr. Jana Aguirre for their technical helpful and writing assistance, as well as Antonio Gil, Cecilia Dall‘Anese, Hermann Hernández,
Fernando Gil Catalinas, and Marta Artamendi all members of the Department of Nephrology of San Pedro Hospital, for their collaboration.

Availability of data and materials
The datasets supporting the conclusions of this article are available in the ClinVar repository, http://www.ncbi.nlm.nih.gov/clinvar/.

Authors’ contributions
CCA performed the molecular genetic studies, the data analysis and the genotype-phenotype correlation supervised by EDG. ACL was involved in the collection of the samples and all the clinical and histological information, and EHL and MSC carried out renal pathological analysis. All authors read and approved the final manuscript.

Funding
No funding to declare.

Ethics approval and consent to participate
This study was conducted according to protocols approved by the Ethical Committee of La Rioja (CEICLAR PI-161), and written informed consent was obtained from each patient.

Consent for publication
All participants signed a consent form for publication. None of family members were under 16 years of age.

Competing interests
All the authors declared that they have no competing interests.

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Author details
1Molecular Diagnostics Unit, Center for Biomedical Research (CIBIR), Fundación Rioja Salud, C/Piqueras 98, 26006 Logroño, La Rioja, Spain.
2Department of Nephrology, San Pedro Hospital, Logroño, La Rioja, Spain.
3Molecular Diagnostics Unit, Center for Biomedical Research (CIBIR), La Rioja Salud, C/Piqueras 98, 26006 Logroño, La Rioja, Spain.
4Department of Nephrology, San Pedro Hospital, Logroño, La Rioja, Spain.

Received: 2 March 2016 Accepted: 25 September 2017
Published online: 31 October 2017

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