University of Siena

Ph.D in Medical Genetics

Autosomal Alport Syndrome: a new model including both dominant and recessive inheritance

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….to my family

1.INTRODUCTION

1. INTRODUCTION

1.1 Alport syndrome

1.1.1 Alport Syndrome

Alport syndrome (ATS) is a nephropathy characterized by the association of progressive hematuric nephritis with ultrastructural changes of the glomerular basement membrane (thinning, thickening and splitting), sensorineural deafness, and variable ocular abnormalities (anterior lenticonus, macular flecks and cataracts).[1,2] ATS accounts for 1-2% of all patients who start renal replacement therapy in Europe, with an estimate frequency of about 1 in 5000.[3,4] ATS is characterized by changes of type IV collagen α3, α4 and α5 network of GBM.[5] These proteins are encoded by three genes: *COL4A3* and *COL4A4* which are located head-to-head on chromosome 2, and *COL4A5* which is located on the long arm of the X chromosome, head-to-head with another type IV collagen gene, *COL4A6*, which encodes the α6(IV) chain, not expressed in the GBM. *COL4A6* gene has been shown to contain two alternative first exons and a huge second intron.

X-linked inheritance, due to *COL4A5* mutations, is the most common mode of transmission (XLAS, OMIM 301050). In this form, 70% of affected males reach end stage renal disease (ESRD) before 30 years (juvenile form), while only few cases (30%) progress toward ESRD after 30 years (rare adult form).[6] The prognosis of X-linked ATS is usually regarded as favourable in females. Usually, microhaematuria is the cardinal feature of the disease in females, although the risk of progression to end-stage renal disease appears to increase after 60 years of age.[4]

X-linked ATS is rarely associated with diffuse leiomyomatosis (ATS-DL), a benign hypertrophy of the visceral smooth muscle in gastrointestinal, respiratory

and female reproductive tracts. All patients with the ATS-DL complex have been found to have deletions that encompass the 5' ends of the *COL4A5* and *COL4A6* genes and include the bidirectional promoter. Unlike the *COL4A5* breakpoint, whose position varies among different patients, the *COL4A6* breakpoint is consistently found within intron II.[7,8]

The autosomal recessive (ARAS, OMIM 203780) form of the disease is due to mutations in the *COL4A3* and *COL4A4* genes, located in 2q36-37, and is reported in 15% of families in European countries.[9,10] Autosomal recessive transmission due to *COL4A3* and *COL4A4* mutations is suggested by the presence of one of the following features: i) Severe early disease in both females and males, both reaching ESRD in the first or second decade of life; ii) Absence of severe signs in parents (they may be completely asymptomatic or may have isolated microhematuria); iii) parental consanguinity. The autosomal dominant form of ATS has been described more recently (ATS-AD, OMIM 104200).

The existence of a pure autosomal-dominant form of ATS (OMIM #104200) has been questioned for decades. Feingold *et al.*[11] presented convincing evidence of an autosomal-dominant form of ATS in their paper, and an autosomal-dominant form of ATS was one of the six types of the disease in the provisional classification system proposed by Atkin, Gregory, and Border.[12] In 2000, the molecular basis of this form has been clarified.[13] Heterozygous mutations in *COL4A4* or *COL4A3* genes have been found also in subset of patients with "Benign Familial Hematuria" (BFH, OMIM 141200) or "Thin Basement Membrane Nephropathy" (TBMN). BFH is clinically defined by persistent glomerular hematuria and by the absence of extra-renal findings.[14,15]

1.1.2 Pathogenesis

Basement membranes are ubiquitous sheet-like extracellular structures separating cells of organized tissues from the interstitial stroma. The basement membranes play a role in cell adhesion and differentiation and also in tissue regeneration. The basement membrane of renal glomeruli (GBM) is unique and forms a well-defined layer located between endothelial cells and the epithelial podocytes where it functions as a size selective sieve of macromolecules.

Alport syndrome is characterized by abnormal GBM structure and hematuria resulting from mutations in the genes of type IV collagen. Type IV collagen is the major structural component of basement membranes where it forms the structural meshwork.[16]

In humans, six distinct α chains of type IV collagen designated α1(IV)-α6(IV) have been identified each encoded by a different gene designated *COL4A1-COL4A6*, respectively. These genes are large and complex, each comprising ~50 exons. They are paired in a head to head fashion with the *COL4A1* and *COL4A2* genes on chromosome 13q23, *COL4A3* and *COL4A4* on chromosome 2q36-37 and *COL4A5* and *COL4A6* on chromosome Xq22.3. (Figure 1)

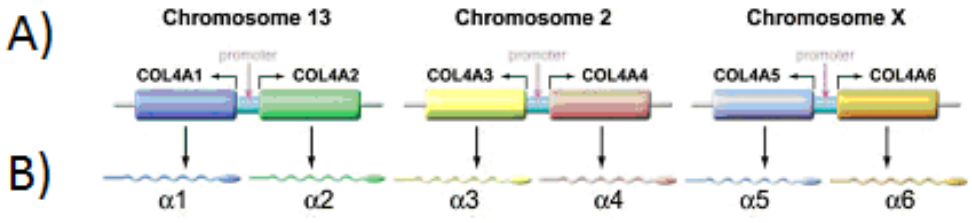

Figure 1. Type IV collagen genes, α chains, and GBM specific isoforms (Tryggvason 2006). (A) The six collagen IV genes (*COL4A1* to *COL4A6*) located pairwise in a head-to-head manner on three different chromosomes generate six different α chains that have a globular noncollagenous domain at their C-terminus (B).

The primary structure of these chains is similar, each chain having a carboxyl-terminal noncollagenous domain (NC1) of ~230 amino acid residues, an ~1400-residue collagenous region that forms the triple helix together with two other a-chains, and an ~25-residue noncollagenous sequence at the amino terminus (7S).

Each collagen molecule is formed from three α-chains. The NC1 domain initiates the assembly and governs the process of α-chains selection. In type IV collagen the Gly-Xaa-Yaa-repeat collagenous domain is frequently interrupted by noncollagenous sequences which give flexibility to the triple helix and the basement membrane meshwork (Figure 2).[5]

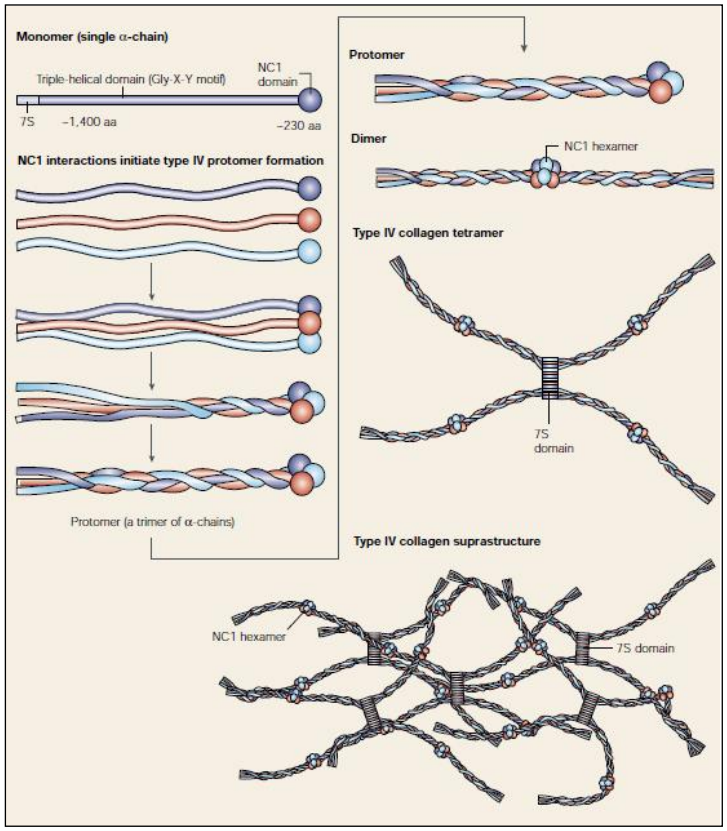

Figure 2: Structure of type IV collagen (Kalluri R, 2003)

Only three combinations of the 6 different α-chains occur: $\alpha1_2\alpha2$, $\alpha3\alpha4\alpha5$ and $\alpha5_2\alpha6$. Collagen molecules (protomers) are then secreted whereupon they

self–assemble at the amino terminal forming tetramers and at the C-terminal forming dimers. Only three types of type IV collagen netwoks are known to exist: $\alpha1_2/\alpha2$ protomers bridge to themselves forming the $\alpha1/\alpha2$ network; $\alpha3/\alpha4/\alpha5$ protomers bridge to themselves forming the $\alpha3/\alpha4/\alpha5$ network; $\alpha1_2\alpha2$ protomers bridge to $\alpha5_2\alpha6$ promoters forming the $\alpha1/\alpha2/\alpha5/\alpha6$ network.

The $\alpha1/\alpha2$ network is ubiquitous in basement membranes whereas the other two networks show a restricted distribution that presumably reflects function. The $\alpha3/\alpha4/\alpha5$ network is prominent in sites that serve as filtration barriers, whereas $\alpha1/\alpha2/\alpha5/\alpha6$ network is often found in basement membranes that undergo repeated stretching. The $\alpha3/\alpha4/\alpha5$ network is predominant one in the glomerular basement membrane (GMB) as well as in several basement membranes in the eye and inner ear. The $\alpha1/\alpha2/\alpha5/\alpha6$ network is expressed in Bowman's capsule of the glomerulus and in basement membranes surrounding smooth muscle cells of vessels and viscera. This network is also present in subepithelial basement membrane of viscera and epidermis. In Goodpasture syndrome, an autoimmune disease characterized by hematuria and pulmonary hemorrhage, the main epitope for autoantibodies have been localized to the NC1 domain of the α 3(IV) chain but recently also against the α 4(IV) chain. [17,18]

1.1.3- Diagnosis and treatment

The diagnosis of collagen IV-related nephropathies rests on (1) clinical history and physical examination, which may include audiologic and ophthalmic evaluation; (2) detailed family history and possibly urinalyses on first- and second-degree relatives; (3) immunohistochemical analysis of basement membrane type IV collagen expression, using skin and/or renal biopsy specimens; and (4) examination of renal biopsy specimens by electron microscopy. With these tools, the diagnosis can be confirmed in most cases. Molecular genetic testing of the type IV collagen genes *COL4A3*, *COL4A4*, and *COL4A5* is available on a clinical basis.

Treatment of manifestations: angiotensin-converting enzyme inhibitor and/or angiotensin receptor blocker in proteinuric individuals; routine treatment of hypertension; dialysis and renal transplantation for ESRD; routine treatment of sensorineural hearing loss and cataracts; surgical intervention for symptomatic leiomyomas. Prevention of secondary complications: Protect corneas of those with recurrent corneal erosions from minor trauma.

Surveillance: follow-up of all individuals with a collagen IV-related nephropathy with a nephrologist; monitor females with XLAS with measurement of blood pressure and renal function; audiologic evaluation of children every one to two years beginning at age six to seven years; monitor transplant recipients for development of anti-glomerular basement membrane antibody-mediated glomerulonephritis.

Testing of relatives at risk: Evaluate at-risk family members either by urinalysis or, if the disease-causing mutation(s) in the family are known, by molecular genetic testing.

1.2 Genetics of Alport syndorme :

1.2.1 X-linked Alport syndrome (ATS -XL)

X-linked Alport syndrome accounts for ~85% of all cases and arises from mutations in the *COL4A5* gene.[19] Over 350 different mutations have been reported including large deletions, missense and nonsense mutations, small deletions/insertions causing frameschifts, and splice site mutations.[6] No mutational "hot spot" are know. With few exception, each family carriers a unique mutation, but up to 18% of cases are de novo mutations.

1.2.2 Autosomal Alport syndrome: recessive (ATS-AR) and dominant (ATS-AD)

Autosomal-recessive Alport syndrome accounts for ~15% of cases and results from homozygous or compound heterozygous mutations in *COL4A3* or *COL4A4* genes.[20] Over 40 different mutations in these genes have been identified with the same spectrum of mutation as for *COL4A5*. Rare examples of autosomal dominant Alport syndrome have been reported, caused by a mutation in either the *COL4A3* or *COL4A4* gene.[21]

1.2.3 "Benign Familial Hematuria" or "Thin Basement Membrane Nephropathy" (TBMN)

Thin glomerular basement membrane disease (TBMD) is a hereditary nephropathy characterized by thinning of the glomerular basement membrane evinced by electron microscopy and, clinically, by isolated hematuria without extrarenal manifestations. Familial aggregation is found in 50-60% of cases, with autosomal dominant transmission. TBMD is considered to belong to the type IV collagen spectrum of diseases, since heterozygous mutations of the *COL4A3* or *COL4A4* gene have been detected in more than 30% of patients. The disease is found in 1-2% of biopsies, but the prevalence in the general population may be higher. The differential diagnosis with Alport's syndrome may be difficult and

requires accurate family investigations, immunohistochemical evaluation of type IV collagen alpha chains in renal tissue and, if appropriate, genetic studies. Progression towards chronic renal failure, although rare, has been reported in some patients, and may be related to the phenotypical variability of *COL4A3/COL4A4* mutations, to a missed Alport syndrome, or to superimposed glomerular disease. Patients suffering from TBMD and affected relatives should be periodically examined for signs of disease progression and informed about the possibility of transmitting the autosomal recessive form of Alport's syndrome.[22]

1.2.4 Alport Syndrome and leiomyomatosis

XLAS is sometimes associated with diffuse leiomyomatosis (DL), a benign hypertrophy of the visceral smooth muscle in gastrointestinal, respiratory and female reproductive tracts.[7] The esophageal wall is typically involved and it causes dysphagia, post-prandial vomiting, retrosternal or epigastric pain since late childhood. Affected females typically exhibit genital leiomyomas, with clitoral hypertrophy and variable involvement of the labia majora and uterus. Bilateral cataracts also occur frequently in affected individuals. Periurethral and perirectal areas are involved less frequently.[23] The symptoms of leiomyomatosis are equally severe in females and males. This suggests that leiomyomatosis is fully expressed in females, with complete penetrance, in contrast to the manifestations of renal disease, which are in general more pronounced in men.[24] In the literature, all patients with the Alport Syndrome – Diffuse leiomyomatosis (ATS-DL) complex have been found to have deletions that encompass the 5' ends of the *COL4A5* and *COL4A6* genes and include the bidirectional promoter.[25] Unlike the *COL4A5* breakpoint, whose position varies among different patients, the *COL4A6* breakpoint is consistently found within intron II .[7,8]

2.RESULTS

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2.1. Results overview

My research project focused on the analysis of the *COL4A4* and *COL4A3* genes in a large cohort of patients, using DHPLC followed by automated sequencing of exons with altered profiles.

I analyzed 148 patients: 71 patients for *COL4A3* and *COL4A4* genes, 73 for *COL4A4* gene and 4 for *COL4A3* gene. Molecular analysis revealed in 25 cases *COL4A4 or COL4A3* gene mutations: 15 autosomal dominant forms (from 15 different families) and 9 autosomal recessive forms (from 7 different families) and 1 autosomal dominant and recessive (Table 1).

Patients' code	Family' Code	Nucleotide Change	Effect on Coding Sequence	Gene	References of mutations
#2663	GLS	4001G>A	G1334E	*COL4A3*	Previously reported by Heidet, et al: J Am Soc Nephrol 2001
#50	LAZ	3574G>A	G1192R	*COL4A3*	Unpublisched data
#2718	WEI	1933-1934insG 4802-4804delT	R645fsX690 P1601fsX1614	*COL4A3* *COL4A3*	Unpublished data Unpublished data
#2003	GRE	IVS19-7T>G	IVS19-7T>G	*COL4A3*	Unpublished data
#2064	MCI	1900G>T	G634X	*COL4A3*	Unpublished data
#3337	FEL	3134G>T	G1045V	*COL4A3*	Previously reported by Pescucci C, et al: Kidney Int 2004
#2740	STI	3134G>T	G1045V	*COL4A3*	Previously reported by Pescucci C, et al: Kidney Int 2004
#2370	DAG	IVS28+2T>G	IVS28+2T>G	*COL4A4*	Marcocci E, et al: Nephrol Dial Transplant 2009
#2417	MVA	1884-1886delC	P629fsX652	*COL4A4*	Marcocci E, et al: Nephrol Dial Transplant 2009
#2456	MCF	2279-2280insG	R761fsX786	*COL4A4*	Longo I, et al: Nephrol Dial Transplant 2006
#2650	PLT	2590G>A 104A>G	G864R Y35C	*COL4A4* *COL4A4*	Longo I, et al: Nephrol Dial Transplant 2006 Longo I, et al: Nephrol Dial Transplant 2006

#2724	EVI	4493-4495delG	G1498fsX1551	*COL4A4*	Marcocci E, et al: Nephrol Dial Transplant 2009
#2777	FIG	2374G>A	G792R	*COL4A4*	Longo I, et al: Nephrol Dial Transplant 2006
#2441	PUX	940G>T	G314C	*COL4A4*	Marcocci E, et al: Nephrol Dial Transplant 2009
#570	FRI	IVS35+1 G>A	IVS35+1 G>A	*COL4A4*	Marcocci E, et al: Nephrol Dial Transplant 2009
#2937	MRM	1579-1581delG	G527fsX652	*COL4A4*	Marcocci E, et al: Nephrol Dial Transplant 2009
#2992	LRC	1579G>T	G527C	*COL4A4*	Marcocci E, et al: Nephrol Dial Transplant 2009
#2907	PZZ	1884-1886delC	P629fsX652	*COL4A4*	Previously reported by Marcocci E, et al: Nephrol Dial Transplant 2009
#2995	GEI	1837G>A	G613R	*COL4A4*	Marcocci E, et al: Nephrol Dial Transplant 2009
#2964	DIL	4749-4752delTC	1583fsX1632	*COL4A4*	Unpublished data
#2969	BAC	4129C>T	R1377X	*COL4A4*	Previously reported by Boye et al: Am J Hum Genet 1998
#H381	UTT	508G>A	G170R	*COL4A4*	Unpublished data
#2530	BRD	4900T>C	C1634S	*COL4A4*	Pescucci C, et al: Kidney Int 2004

Table 1: *COL4A3* or *COL4A4* gene mutations

In my PhD study I contributed to the analysis of families with ATS-AR reported *by Longo I, et al: Nephrol Dial Transplant 2006* and I personally analyzed 8 families with ATS-AD. This study allowed me to publish an article as first author in Nephrology Dialysis Transplantation (2.2 Results). In the last year of my PhD study I also contributed to the analysis of three families with ATS-DL (2.3 Results). Recently, I identified one mutation in the *COL4A3* gene in an interesting ATS family (STI) where both dominant and recessive inheritance is present (2.4 Results).

During the course of my PhD studies, interestingly, I have found the single base deletion p.P629fsX652 in two different unrelated families coming from Trapani, and the missense mutation p.G1045V, previously reported by *Pescucci et al, 2004* (Family 3, come from Treviso), in other two different

unrelated families coming from Treviso (Figure 3). This may suggest a possible founder effect for these two mutations.

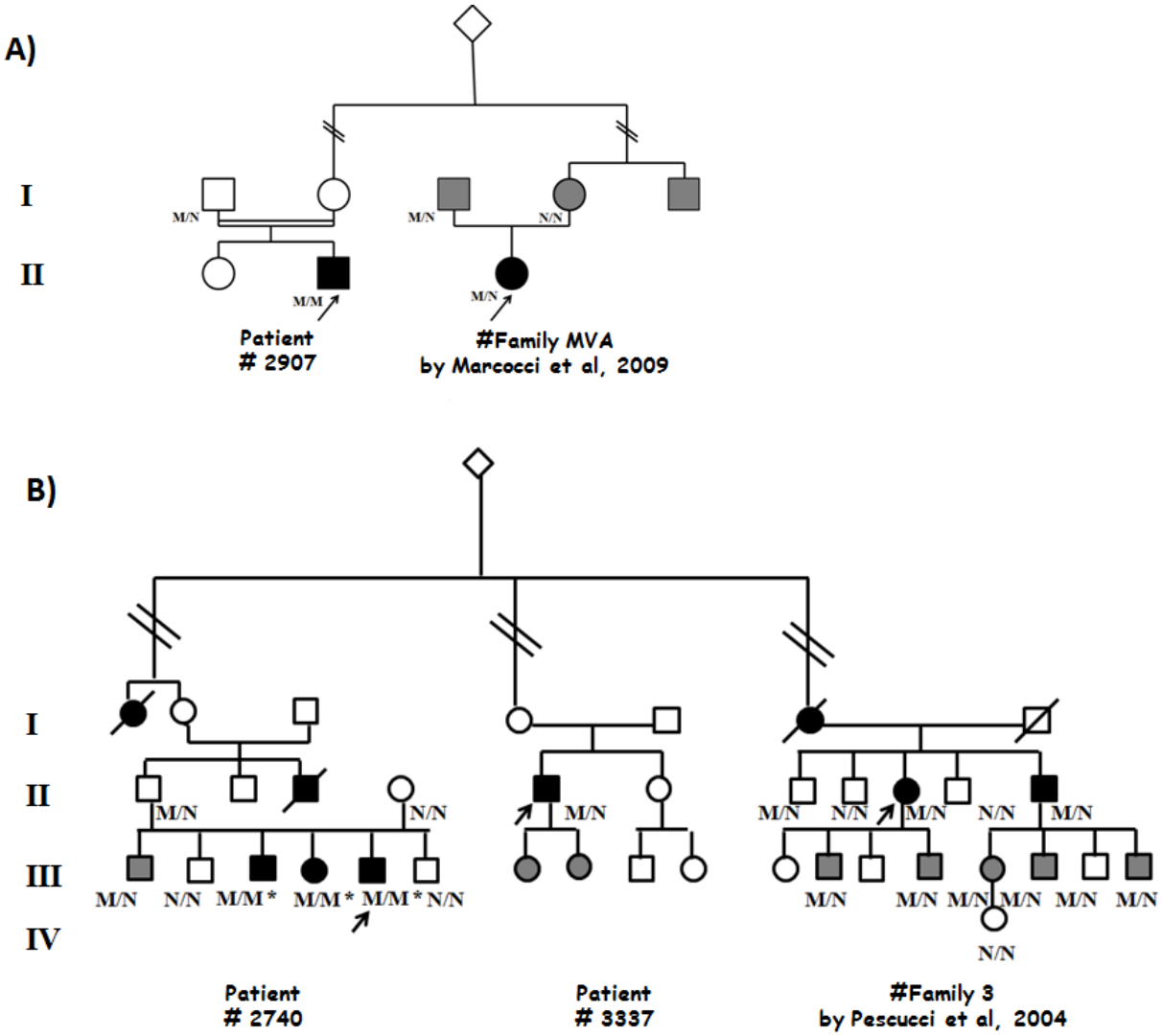

Figure 3. A) Pedigree of two different unrelated families coming from Trapani; B) Pedigree of three different unrelated families coming from Treviso: Open squares are males and open circles are females. Filled grey symbols are individuals with microhemauturia. Filled black symbols indicate individuals with microhematuria, macrohematuria or hearing loss or renal failure. White symbols indicate individuals without clinical sings of the disease. The arrows indicate the index proband. The genotype at *COL4A3* locus is indicated below each symbol as follows: N=wild type allele; M=mutated allele; M*= allele with a second hypothetical mutation.

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2.1.1 Materials and Methods

Patients collection

Since the beginning of my PhD program 148 patients with a possible diagnosis of autosomal ATS have been collected in the Medical Genetics Unit of the University Hospital of Siena.

Molecular analysis

Blood samples were collected from patients after informed consent. Genomic DNA was isolated using QIAamp DNA blood maxi kit, according to the manufactures' protocol (Quiagen, Hilden, Germany). All the *COL4A3* and *COL4A4* exons were amplified using these polymerase chain reaction (PCR) condition: genomic DNA (50 ng/µl) was PCR amplified in 50 µl containing 0.5 pM/µl of each primer, 2 mM dNTPs, 1x PCR Gold Buffer, 1.5mM $MgCl_2$, and 0,02 U/µl of Amply Taq Gold. PCR cycles were as follows: 95°C for 5 min, followed by 35 cycles consisting of 95°C for 1 min, annealing for 1 min, and 72°C for 1 minute, ended by a final extension at 72°C for 5 min and get of the original strand of DNA. Mutation analysis was performed by denaturing high performance liquid chromatography (DHPLC) using Transgenomic WAVE ™ (Transegenomic, San Jose, CA, USA). PCR products were denatured at 95°C, re-annealed at 65°C for 10 min and cooled at 4°C to generate heteroduplex.[21] The optimal column temperature for fragments analysis was calculated using the WaveMaker Software (Transgenomic, San Jose, CA, USA). PCR products resulting in abnormal DHPLC profiles were purified and sequenced on both strands by using PE Big dye terminator cycle sequencing kit on an ABI Prism 310 genetic analyser (PE Applied Biosystems, Foster City, CA, USA).

2.2

Autosomal dominant Alport syndrome: molecular analysis of the *COL4A4* gene and clinical outcome.

Marcocci E, Uliana V, Bruttini M, Artuso R, Silengo MC, Zerial M, Bergesio F, Amoroso A, Savoldi S, Pennesi M, Giachino D, Rombolà G, Fogazzi GB, Rosatelli C, Martinhago CD, Carmellini M, Mancini R, Di Costanzo G, Longo I, Renieri A, Mari F.

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Autosomal dominant Alport syndrome: molecular analysis of the *COL4A4* gene and clinical outcome

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Abstract

Background. Alport syndrome is a clinically and genetically heterogeneous nephropathy characterized by glomerular basement membrane lesions often associated with hearing loss and ocular anomalies. While the X-linked and the autosomal recessive forms are well known, the autosomal dominant form is not well acknowledged.

Methods. We have clinically investigated 38 patients with a diagnosis of autosomal dominant Alport syndrome belonging to eight different families. The analysis of the *COL4A4* gene was performed by denaturing high performance liquid chromatography and automated DNA sequencing.

Results. In our cohort of patients, only 24.3% (9/37) reached end-stage renal disease, at the mean age of 51.2 years. Four patients had hearing loss (13.3%) and none ocular changes. Molecular analysis revealed eight novel private *COL4A4* gene mutations: three frameshift, three missense and two splice-site mutations.

Conclusions. These data indicate autosomal dominant Alport syndrome as a disease with a low risk of ocular and hearing anomalies but with a significant risk to develop renal failure although at an older age than the X-linked form. We were unable to demonstrate a genotype–phenotype correlation. Altogether, these data make difficult the differential diagnosis with the benign familial haematuria due to heterozygous mutations of *COL4A4* and *COL4A3*, especially in young patients, and with the X-linked form of Alport syndrome in families where only females are affected. A correct diagnosis and prognosis is based on a comprehensive clinical investigation in as many family members as possible associated with a broadly formal genetic analysis of the pedigree.

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Keywords: ADAS; ATS; autosomal dominant Alport syndrome; BFH; *COL4A4*; TBMN

Introduction

Alport syndrome (ATS) is a progressive heterogeneous nephropathy characterized by the association of progressive haematuric nephritis with ultrastructural changes of the glomerular basement membrane (GBM), sensorineural deafness and variable ocular abnormalities [1–3]. ATS accounts for 1–2% of all patients who start renal replacement therapy in Europe, with an estimate frequency of about 1 in 5000 [4–6].

ATS is characterized by changes of type IV collagen α3, α4 and α5 network of the GBM, and three modes of inheritance are known. The X-linked inheritance, due to mutations in *COL4A5* located in Xq22.3, is the most common mode of transmission (XLAS, OMIM 301050). In this form, 70% of affected males reach end-stage renal disease (ESRD) before 30 years (juvenile form), while only few cases (30%) progress towards ESRD after 30 years (rare adult form) [4,5,7]. Usually, females have only microhaematuria; however, a small percentage of females can develop renal failure. The autosomal recessive (ARAS, OMIM 203780) form of the disease is due to mutations in the *COL4A3* and *COL4A4* genes, located in 2q36–37, and is reported in 15% of families in European countries [8–10]. Autosomal recessive transmission due to *COL4A3* and *COL4A4* mutations is suggested by the presence of one of the following features: (i) severe early disease in both females and males; (ii) absence of severe signs in parents (they may be completely asymptomatic or may have isolated microhaematuria); (iii) parental consanguinity.

The autosomal dominant form of ATS has been described more recently (ADAS, OMIM 104200). In 1997, this form had been linked to the COL4A3/COL4A4 locus in a large family from Northern Ireland and 3 years later there came the first report of a *COL4A3* heterozygous mutation segregating in a family with ADAS [11,12]. Till now, only nine families with ADAS and a proven *COL4A4* or *COL4A3* mutation have been reported. Both female and male patients showed a high clinical variability with a renal phenotype ranging from isolated haematuria to late onset ESRD, associated in few instances with hearing loss [11–15].

Heterozygous mutations in *COL4A4* or *COL4A3* genes have been found also in the subset of patients with 'Benign Familial Hematuria' (BFH, OMIM 141200). BFH is clinically defined by persistent glomerular haematuria and by the absence of extra-renal findings [16–18]. The typical ultrastructural findings are diffuse thinning of GBM.

Although the term 'Thin Basement Membrane Nephropathy' (TBMN) is a pure morphological definition, it is still erroneously used to describe patients with the diagnosis of BFH. In fact, the finding of isolated thinning of the basement membrane can represent the only lesion identified in patients with ATS [19].

In this study, we present eight ATS families with autosomal dominant transmission and heterozygous mutations in the *COL4A4* gene. The aim of this study is to better define the natural clinical history of ADAS in order to help in the differential diagnosis with the other known forms of ATS and with BFH.

Subjects and methods

Patients

Thirty-eight patients belonging to eight families with a clinical suspect of ADAS were enrolled in this study. Patients were diagnosed by experienced nephrologists and clinical geneticists. Urinalysis and renal function evaluations were performed in all patients. Audiological and ophthalmic examinations were performed in several patients. Whenever indicated, a renal biopsy with ultrastructural study by electron microscopy was performed.

Mutation analysis

Blood samples were collected from patients after informed consent. Genomic DNA was extracted using a QIAamp DNA blood maxi kit, according to the manufacturers' protocol (Quiagen, Hilden, Germany). All the *COL4A4* exons were amplified using the primers and PCR condition already described [14]. Mutation analysis was performed by denaturing high performance liquid chromatography (DHPLC) using Transgenomic WAVE™ (Transgenomic, San Jose, CA, USA) and by subsequent genomic sequencing analysis. PCR products were denatured at 95°C, reannealed at 65°C for 10 min and cooled at 4°C to generate heteroduplex [15]. The optimal column temperature for fragments analysis was calculated using the WaveMaker Software (Transgenomic, San Jose, CA, USA). DHPLC analysis was performed at the melting temperature of 60°C for exon 25, 60.2°C for exon 22, 60.6°C for exon 28, 57.7°C for exon 46, 55.3°C for exon 35 and 54°C for exon 16. PCR products resulting in abnormal DHPLC profiles were purified and sequenced on both strands by using a PE Big dye terminator cycle sequencing kit on an ABI Prism 310 genetic analyser (PE Applied Biosystems, Foster City, CA, USA). Segregation analysis was performed by direct sequencing in all families.

Results

A *COL4A4* mutation in eight families with ADAS has been identified by DHPLC analysis (Figure 1). Detailed clinical information of a total of 38 patients belonging to these eight families has been collected (Table 1). The molecular diagnosis was confirmed in 29/38 patients. The remaining nine patients were either deceased or unavailable.

COL4A4 mutations

The eight identified pathogenic mutations were all in a heterozygous state, private and previously not described (Supplementary Figure 1). Seven mutations were distributed in the collagenic domain, and one mutation was localized in the C-terminal domain (Figure 2a). Three were

Table 1. Clinical and molecular data of reported patients

Family/subject	Sex	Age	Urinalysis	Renal function	GBM abnormalities	Ophthalmic examination	Audiological examination	*COL4A4* heterozygous mutation			*COL4A4* heterozygous polymorphisms
								Nucleotide change	Effect on coding sequence	Exon/intron	
MVA/I1	M	55	Microhaematuria	Normal	n.d.	Normal	Mild h.l.	1884–1886del C	P629fsX652	Exon 25	1, 4, 5, 11
MVA/II1	F	25	Microhaematuria proteinuria	Normal	Thinning, thickening	Normal	Normal	1884–1886del C	P629fsX652	Exon 25	1, 4, 6, 7, 8, 9, 10, 12
MRM/II3	M	43	Microhaematuria proteinuria	CRF at 40 year	Thickening	Normal	Normal	1579–1581del G	G527fsX652	Exon 22	None
MRM/III1	F	15	Microhaematuria proteinuria	Normal	n.d.	Normal	Normal	1579–1581del G	G527fsX652	Exon 22	None
MRM/I1	F	50^	Microhaematuria	ESRD at 46 year	n.d.	Normal	Normal	n.a.	n.a.	n.a.	n.a.
MRM/I2	M	68^	Microhaematuria	ESRD at 67 year	n.d.	Normal	s.h.l.	n.a.	n.a.	n.a.	n.a.
MRM/II1	M	44	Microhaematuria proteinuria	Normal	n.d.	Normal	n.d.	n.a.	n.a.	n.a.	n.a.
MRM/II4	F	40	Microhaematuria proteinuria	Normal	n.d.	Normal	n.d.	n.a.	n.a.	n.a.	n.a.
EVI/II2	M	33	Microhaematuria proteinuria	Normal	Thinning	Normal	c.h.l.	4493–4495 del G	G1498fsX1551	Exon 46	5, 6, 7, 8, 9, 10, 11, 12
EVI/I2	M	46^	Microhaematuria proteinuria	ESRD at 42 year	n.d.	Normal	Normal	n.a.	n.a.	n.a.	n.a.
EVI/I3	M	60^	Microhaematuria proteinuria	n.d.	n.d.	Normal	Normal	n.a.	n.a.	n.a.	n.a.
GEI/III2	F	7	Microhaematuria	Normal	n.d.	Normal	Normal	1837G>A	G613R	Exon 25	3, 4, 6, 7, 11, 12
GEI/II2	M	45	Microhaematuria	Normal	n.d.	Normal	Mild h.l.	1837G>A	G613R	Exon 25	2, 5, 6, 7, 8, 9, 10, 11, 12
GEI/I1	F	60^	n.d.	ESRD	n.d.	n.d.	n.d.	n.a.	n.a.	n.a.	n.a.
GEI/II4	F	41	Microhaematuria	Normal	n.d.	n.d.	n.d.	1837G>A	G613R	Exon 25	2, 5, 6, 7, 8, 9, 10, 11, 12
PUX/I1	F	n.d.	n.d.	ESRD	n.d.	n.d.	n.d.	940G>T	G314C	Exon 16	None
PUX/II1	M	48	Microhaematuria proteinuria	Normal	Thinning, splitting	n.d.	n.d.	940G>T	G314C	Exon 16	6, 7, 8, 9, 10, 11, 12
PUX/III1	F	7	Microhaematuria	Normal	n.d.	Normal	Normal	940G>T	G314C	Exon 16	None
LCR/I2	M	76	Microhaematuria	Normal	n.d.	Normal	s.h.l.	1579G>T	G527C	Exon 22	2, 5, 6, 7, 8, 9, 10, 11, 12
LCR/II1	F	47	Microhaematuria	Normal	n.d.	Normal	Normal	1579G>T	G527C	Exon 22	None
LCR/II3	M	40	Microhaematuria	Normal	n.d.	Normal	Normal	1579G>T	G527C	Exon 22	2, 5, 6, 7, 8, 9, 10, 11, 12
LCR/III1	F	26	Micro-macrohaematuria proteinuria	Normal	Thinning, thickening, splitting	Normal	Normal	1579G>T	G527C	Exon 22	8, 9,

Continued

Table 1. *Continued*

Family/subject	Sex	Age	Urinalysis	Renal function	GBM abnormalities	Ophthalmic examination	Audiological examination	*COL4A4* heterozygous mutation			*COL4A4* heterozygous polymorphisms
								Nucleotide change	Effect on coding sequence	Exon/intron	
LCR/III2	F	21	Micro-macrohaematuria	Normal	Thinning, thickening, splitting	Normal	Normal	1579G>T	G527C	Exon 22	None
LCR/III3	M	11	Microhaematuria	Normal	n.d.	Normal	Normal	1579G>T	G527C	Exon 22	2, 6, 7, 8, 9, 10, 11, 12
DAG/I1	M	73	Microhaematuria proteinuria	ESRD at 60 year	n.d.	Normal	Normal	IVS28+2 T>G	IVS28+2 T>G	Intron 28	4, 6, 7, 8, 9, 10, 11, 12
DAG/II1	M	49	Microhaematuria proteinuria	CRF	Thinning	n.d.	Normal	IVS28+2 T>G	IVS28+2 T>G	Intron 28	2, 4, 5,
DAG/III1	M	20	Microhaematuria	Normal	n.d.	n.d.	n.d.	IVS28+2 T>G	IVS28+2 T>G	Intron 28	4, 6, 7, 8, 9, 10, 11, 12
DAG/III2	F	11	Microhaematuria	Normal	n.d.	n.d.	n.d.	IVS28+2 T>G	IVS28+2 T>G	Intron 28	5
FRI/I2	F	66^	Microhaematuria proteinuria	ESRD	n.d.	Normal	Normal	n.a.	n.a.	n.a.	n.a.
FRI/II2	F	64	Microhaematuria proteinuria	ESRD at 60 year	n.d.	Normal	Normal	IVS35+1G>A	IVS35+1G>A	Intron 35	None
FRI/II3	M	55	Microhaematuria proteinuria	ESRD at 45 year	Thinning, thickening, splitting	Cataract post-tx	Normal	IVS35+1G>A	IVS35+1G>A	Intron 35	2, 5, 6, 7, 8, 9, 10, 11, 12
FRI/III2	M	42	Microhaematuria	Normal	n.d.	Normal	Normal	IVS35+1G>A	IVS35+1G>A	Intron 35	2, 5, 6, 7, 8, 9, 10, 11, 12
FRI/III3	F	40	Microhaematuria proteinuria	Normal	n.d.	Normal	Normal	IVS35+1G>A	IVS35+1G>A	Intron 35	2, 5, 6, 7, 8, 9, 10, 11, 12
FRI/III6	M	34	Microhaematuria proteinuria	CRF	n.d.	Normal	Normal	IVS35+1G>A	IVS35+1G>A	Intron 35	None
FRI/IV1	F	16	Microhaematuria proteinuria	Normal	n.d.	n.d.	Normal	IVS35+1G>A	IVS35+1G>A	Intron 35	2, 5, 6, 7, 10, 11, 12
FRI/IV3	F	11	Microhaematuria	Normal	n.d.	Normal	Normal	IVS35+1G>A	IVS35+1G>A	Intron 35	2, 5, 6, 7, 10, 11, 12
FRI/IV4	M	9	Microhaematuria	Normal	n.d.	Normal	Normal	IVS35+1G>A	IVS35+1G>A	Intron 35	2, 5, 6, 7, 10, 11, 12
FRI/IV5	F	6	Microhaematuria	Normal	n.d.	Normal	Normal	IVS35+1G>A	IVS35+1G>A	Intron 35	2, 6, 7, 10, 11, 12

ESRD: end-stage renal disease; GBM: glomerular basement membrane; c.h.l.: conductive hearing loss; s.h.l.: sensoryneuronal hearing loss; n.d.: not defined; n.a.: DNA not available; ^: deceased; M: male; F: female, GBM: glomerular basement membrane.
Numbers in the last column refer to polymorphisms numbers of the Table 2.

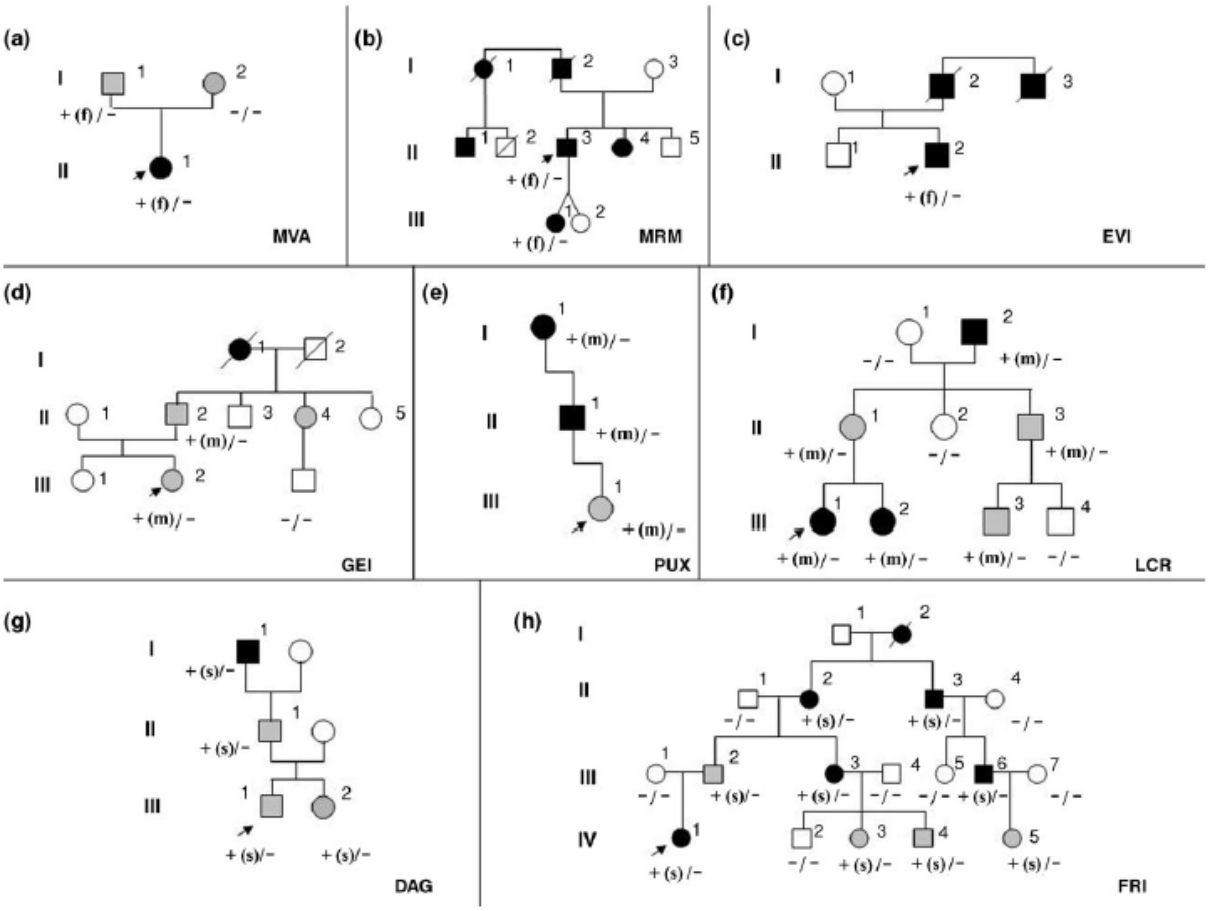

Fig. 1. Pedigree of families. The figure represents the pedigree of family 1 (a), 2 (b), 3 (c), 4 (d), 5 (e), 6 (f), 7 (g), 8 (h). □ Males, ○ females. Filled grey symbols are individuals with isolated microhaematuria. Filled black symbols indicate individuals with microhaematuria plus macrohaematuria or proteinuria or hypoacusia or renal failure. White symbols indicate individuals without clinical sings of the disease. An oblique bar indicates a deceased individual. The arrows indicate the index patients. The genotype at the *COL4A4* locus is indicated below each symbol as follows: −, wild type allele; +, mutated allele. The type of mutation is indicated in brackets: f = frameshift mutation; m = missense mutation; s = splice site mutation. In (a) case I-2 has microhaematuria probably not related to ATS.

frameshift mutations leading to a protein truncation within the collagenous domain (families MVA and MRM) or in the C-terminal domain in one family (family EVI). Three were missense mutations leading to glycine substitutions in the collagenous domain (families GEI, PUX and LCR). Two were splice-site mutations localized in intron 28 and 35 (families DAG and FRI).

During the DHPLC screening, we also identified 11 previously reported polymorphisms and one rare variant (Table 2).

Table 2. Polymorphisms in the *COL4A4* gene

	Nucleotide change	Effect on coding sequence	Exon/intron
1	IVS5+86C>T[a]	IVS5+86C>T[a]	Intron 5
2	1444C>T	P482S	Exon 21
3	1634G>C	G545A	Exon 23
4	IVS28–5C>T	IVS28–5C>T	Intron 28
5	3011T>C	L1004P	Exon 33
6	3594G>A	G1198G	Exon 39
7	3684G>A	K1228K	Exon 39
8	4080A>G	P1360P	Exon 42
9	3979A>G	M1327V	Exon 42
10	4204C>T	P1402S	Exon 44
11	4548A>G	V1516V	Exon 47
12	4932C>T	F1644F	Exon 48

[a]Not reported as polymorphism (rare variant).

Clinical data

Clinical features of the 38 affected individuals are described in Table 1. Their mean age is 34 years (range from 6 to 76 years). Six patients died with ESRD at the mean age of 58.3 years (range, 46–68 years). The main clinical manifestation was microscopic haematuria, which was present in 100% of patients. Gross haematuria occurred in 2/37 patients (5.4%). Proteinuria was present in 18/36 patients with known urinalysis (50%). Renal failure occurred in 12/37 patients (32.4%). Among these, three patients aged 34, 43 and 49 years presented CRF (creatinine 1.48 mg/dl and 1.53 mg/dl in the last two cases). The remaining nine patients developed ESRD: six patients reached ESRD after

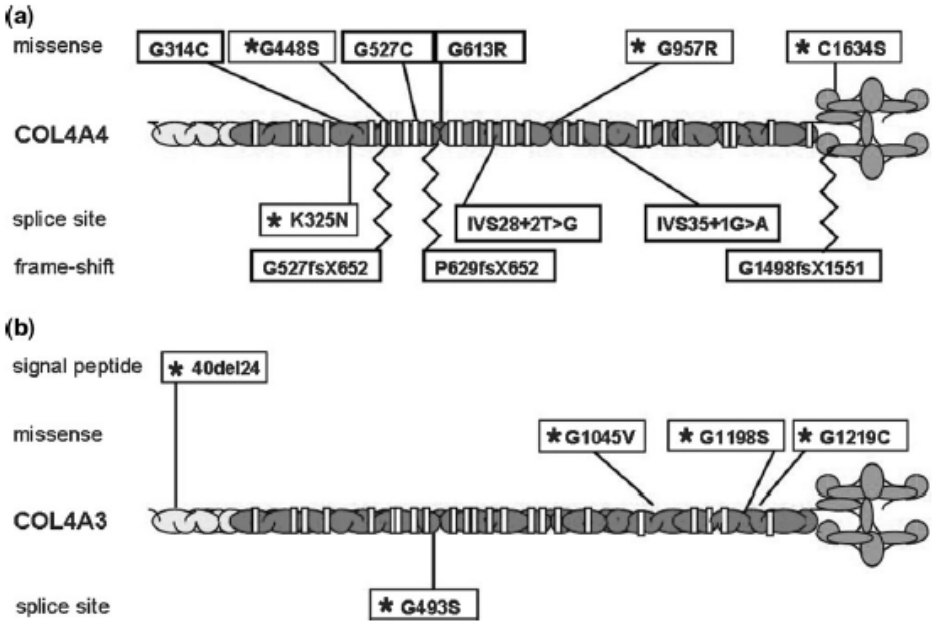

Fig. 2. Distribution of pathogenetic mutations along *COL4A4* (**A**) and *COL4A3* (**B**) genes in ADAS patients. *mutations previously reported as associated with ADAS. All the others are novel mutations reported here.

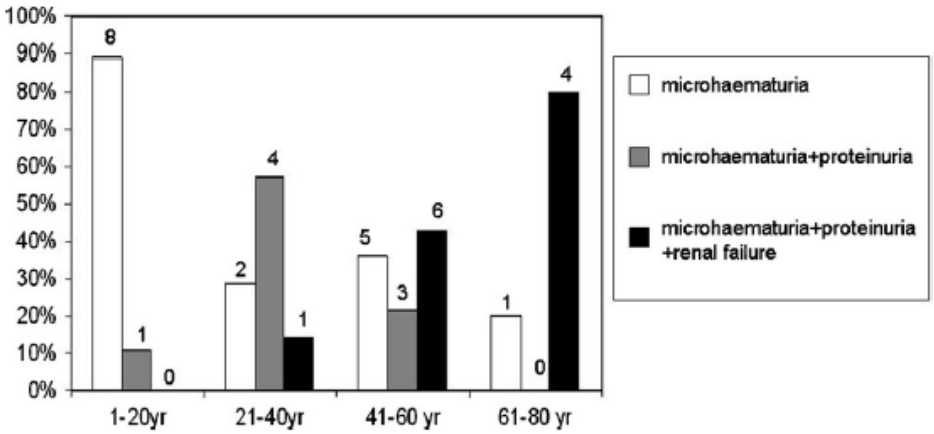

Fig. 3. Graphic representing the phenotype of our 35 patients at different ages. Patients have been divided in four classes of age (0–20; 21–40; 41–60; 61–80) and they have been classified according to their renal involvement. The white bar represents patients with isolated microhaematuria, the grey bar represents cases with microhaematuria and proteinuria and the black bar represents patients with renal failure (both IRC and ESRD). Above each column, the absolute number of patients is reported.

the age of 40 years, at a mean age of 51.2 year (range, 42–67 years), two died at 60 and 66 years of age and for the last patient no clinical data are available.

An ultrastructural examination of the GBM was performed in 8 patients (range of age at renal biopsy from 14 to 43 years) belonging to seven families. Alterations of GBM were identified in all. In six patients, the ultrastructural alterations were clearly diagnostic for ATS, showing a combination of thinning, thickening and splitting of the GBM, while in two patients isolated GBM thinning was identified (EVI-II2, age at biopsy 26 years and DAG II1, age at biopsy 29 years).

Bilateral hearing deficit in the 2000–8000 HZ range, with an onset after 40 years, developed in 4 of 30 tested patients (13.3%). Ocular changes were found in 1 of 30 tested patients (3.3%): this patient, aged 55 years, presented bilateral cataract post-renal transplantation attributed to steroid therapy.

Discussion

ATS and BFH are type IV collagen inherited disorders associated with heterozygous mutations in *COL4A3* and *COL4A4* genes. Till now, only 43 patients belonging to nine families with ADAS have been reported with either *COL4A3* or *COL4A4* mutations [11–15].

In this study, we showed the clinical outcome and the molecular data of 38 patients with ADAS belonging to eight families in whom a pathogenic *COL4A4* mutation was identified (Table 1).

In this series of patients, haematuria, usually microscopic, was present in all cases with known urine analysis, while proteinuria was present in nearly 40% of patients. The development of renal failure was progressive with the age (Figure 3): only one patient (7.1%) younger than 40 years (1/14) presented CRF; two patients among those aged between 41 and 50 years (2/10; 20%) showed renal failure (one patient with ESRD and one patient with CRF); among patients aged between 51 and 60 years, four developed ERSD (4/6; 67%) and four out of five patients older than 60 years developed ESRD (80%).

GBM alterations were identified in all patients in whom a renal biopsy was performed. Only for one family, renal biopsy has not been performed. However, the hypothesis of a diagnosis of ATS is propped by the clinical manifestation of affected members of the family and by the identification of a disease segregating mutation in the *COL4A4* gene leading to a glycine substitution in the collagenous domain of the protein [5]. The ultrastructural study of renal biopsy in almost all patients revealed a combination of thickening, splitting and thinning of the GBM. It is worth noting that two unrelated patients (aged 26 and 29 years) showed isolated thinning of GBM. This observation emphasizes the concept that thinning of the GBM is a non-specific finding and can be predictable of either BFH or ATS [15,19]. Recently, Voskarides K. *et al*. reported eight families with heterozygous *COL4A3* mutations and a phenotype that in some patients was present in isolated haematuria while in older relatives progressed to CRF/ESRD [20]. The only ultrastructural finding in tested patients was a thinning of the GBM, and the authors defined these families as affected by TBMN [20]. Since TBMN is a term often associated with a benign prognosis, these patients could be rather classified as ADAS.

The extra-renal manifestations typical of ATS have not been frequently observed in our cohort of patients. Only 13% of patients developed sensorineural hearing loss that was slowly progressive. The only patient with ocular abnormalities in our series was a 55-year-old male patient who developed bilateral cataract post-renal transplantation, probably due to steroid treatment. The occurrence of congenital or early onset cataract has been already reported although in a minority of patients with a definitive diagnosis of ATS [5].

In all our patients, a heterozygous mutation in the *COL4A4* gene has been identified. These mutations are of different type and are scattered throughout the gene. Heterozygous mutations in *COL4A3* and *COL4A4* are 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 [16,17,21–23]. The intra-familial clinical variability observed in our cohort of patients was mainly due to the age at examination. However, some relatives showed a different clinical outcome at nearly the same age as in the case of family FRI where patient II3 developed ESRD at the age of 45 years while patient III2 showed isolated microhaematuria at the age of 42 years. The intra-familial clinical variability was more evident in families previously reported where even non-penetrant cases have been observed [15]. An inter-familial clinical variability was also present in our cases; in fact, while in nearly all families, older patients developed renal failure, in family LCR a male patient aged 76, and showed normal blood creatinine.

In order to explain such variability, we can hypothesize that the type and/or site of the mutation may be critical, even if genotype–phenotype correlations are made difficult by the fact that the mutations in these genes are often private and there are not mutational hot spots. However, analysing our cases and those already reported, it seems that there is not a clear correlation between type of mutation and phenotype. In fact, mutations of each type (missense, splice site and frameshift) and distributed throughout the genes are reported both in isolated microhaematuria segregating families and in families with progression towards ESRD (ADAS phenotype) (Figure 2a and b). The lack of a genotype–phenotype correlation is also highlighted by the different clinical outcome of patients of the same age, within a family, and thus bearing the same mutation. If the mutation alone cannot explain the clinical variability, we can suppose that the effect of a pathogenic mutation could be influenced by the presence of certain polymorphisms in the same genes and/or that functional variants in other proteins that are key players in renal filtration may act as modifiers.

We have compared the frequency of each sign among our patients and ADAS cases already reported in the literature, considering together patients with *COL4A3* or *COL4A4* gene mutations, given the low number of families reported with mutations in each gene (Table 3) [5,6,12–15]. Haematuria, usually microscopic, is the cardinal feature of the disease and was present in all patients with known urinanalysis, both in our patients and in patients previously reported. Proteinuria was present in about half of cases. Furthermore, our observations confirmed previously reported data about the slow progression towards ESRD and the lower occurrence of extra-renal signs [12–15].

Overall, the clinical outcome of ADAS seems to be close to XLAS in carrier females (Table 3). However, carriers of the XLAS have a higher incidence of proteinuria as presenting feature (75% versus 40–50%), while they have a lower progression towards ESRD than patients with ADAS, being reported in about 18% of females with XLAS [6]. Concerning the extra-renal manifestations, the development of hearing loss is comparable between the two groups (20–30%), while ocular lesions have never been reported in ADAS patients and are present in about 15% of XLAS carriers.

ADAS is suggested by a similar clinical outcome in males and females and vertical transmission of clinical signs. Clinically it is characterized by the presence of microhaematuria, ESRD onset usually after 40 years, ultrastructural changes of the GBM from isolated thinning to a combination of thinning, thickening and splitting, and presence of slowly progressive hearing loss in about 20% of cases. Even if ocular signs have never been reported, a low prevalence in ADAS cannot be excluded.

In conclusion, it is very difficult to predict the prognosis in a patient with a heterozygous mutation in either the *COL4A3* or the *COL4A4* gene. A correct diagnosis and

Table 3. Clinical features of the 38 patients, in comparison with previously reported ADAS [12–15] and XLAS [5,6] patients

	Present study	ADAS literature	ADAS total	XLAS males	XLAS females
Number of patients	38	43	79	218	349
Microhaematuria	100% (38/38)	94.3% (33/35)	97.3% (71/73)	100%	95.5%
Proteinuria	50% (18/36)	41.2% (14/34)	45.7% (32/70)	95%	75.20%
Hearing loss	13.3% (4/30)	27% (10/37)	20.9% (14/67)	79%	28%
Ocular lesions	0/29	0	0	35.2%	15%
ERSD					
Onset: <31 year	0% (0/6)	0% (0/8)	0% (0/14)	76.5%	24%
Onset: 31–40 years	0% (0/6)	12.5% (1/8)	7.1% (1/14)	17.5%	31%
Onset:>40 year	100% (6/6)	87.5% (7/8)	92.8% (13/14)	6%	41%

prognosis is based on a combination of a comprehensive clinical investigation of all family members, including examination of renal and extra-renal signs of ATS in older members, associated with a broadly formal genetic analysis of the pedigree.

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Conflict of interest statement. None declared.

Supplementary data

Supplementary data is available online at http://ndt.oxfordjournals.org.

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2.3

Alport syndrome and leiomyomatosis: description of three cases.

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Alport syndrome and leiomyomatosis: description of three cases

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ABSTRACT

Alport syndrome (ATS) is a nephropathy characterized by the association of progressive hematuric nephritis with ultrastructural changes of the glomerular basement membrane (thinning, thickening and splitting), sensorineural deafness, and variable ocular abnormalities (anterior lenticonus, macular fleckes and cataracts). The most common mode of transmission is X-linked inheritance, due to *COL4A5* mutations. X-linked ATS is rarely associated with diffuse leiomyomatosis (DL), a benign hypertrophy of the visceral smooth muscle in gastrointestinal, respiratory and female reproductive tracts. The ATS-DL complex is due to deletions that encompass the 5' ends of the *COL4A5* and *COL4A6* genes and include the bidirectional promoter. In this paper, we described three ATS-DL cases, two familial and one sporadic bearing a deletion encompassing 5'-end of both *COL4A5* and *COL4A6* genes, identified by MLPA analysis. Array-CGH technique allowed a better definition of deletion size confirming that the proximal breakpoint was within *COL4A6* intron II in two cases. Surprisingly, one case had a deletion extending proximally beyond exon 3 of *COL4A6,* confirmed by qPCR analysis. This in the largest deletion reported to date associated with ATS-DL and this case should lead us to reconsider the mechanisms that may be involved in the development of diffuse leyomiomatosis.

Key words: Alport Syndrome; Diffuse leiomyomatosis; ATS-DL; Array-CGH; MLPA; *COL4A5*; *COL4A6*

Introduction

Alport syndrome (ATS) is a progressive heterogeneous nephropathy characterized by the association of progressive hematuric nephritis with ultrastructural changes of the glomerular basement membrane (thinning, thickening and splitting), sensorineural deafness and variable ocular abnormalities (anterior lenticonus, macular fleckes and cataracts). ATS accounts for 1-2% of all patients who start renal replacement therapy in Europe, with an estimate frequency of about 1 in 5000 [1, 2]. ATS is characterized by an alteration of the type IV collagen α3, α4, α5 network of the glomerular basement membrane (GBM). These proteins are encoded by three genes: *COL4A3* and *COL4A4* which are located head-to-head on chromosome 2, and *COL4A5* which is located on the long arm of the X chromosome, head-to-head with another type IV collagen gene, *COL4A6*, which encodes the α6(IV) chain, not expressed in the GBM. *COL4A6* gene has been shown to contain two alternative first exons and a huge second intron. X-linked inheritance, due to *COL4A5* mutations, is the most common mode of transmission (XLAS, OMIM 301050). In this form, 70% of affected males reach end stage renal disease (ESRD) before 30 years (juvenile form), while only few cases (30%) progress toward ESRD after 30 years (rare adult form) [3]. The prognosis of X-linked ATS is usually regarded as favourable in

females. Usually, microhaematuria is the cardinal feature of the disease in females, although the risk of progression to end-stage renal disease appears to increase after 60 years of age [4]. The autosomal recessive (ARAS, OMIM 203780) and dominant forms (ADAS, OMIM 104200) of the disease are linked to mutations in the *COL4A3* and *COL4A4* genes and are reported in 10-15% of families in European countries [3]. XLAS is sometimes associated with diffuse leiomyomatosis (DL), a benign hypertrophy of the visceral smooth muscle in gastrointestinal, respiratory and female reproductive tracts [5]. The esophageal wall is typically involved and it causes dysphagia, post-prandial vomiting, retrosternal or epigastric pain since late childhood. Affected females typically exhibit genital leiomyomas, with clitoral hypertrophy and variable involvement of the labia majora and uterus. Bilateral cataracts also occur frequently in affected individuals [2]. Periurethral and perirectal areas are involved less frequently [6]. The symptoms of leiomyomatosis are equally severe in females and males. This suggests that leiomyomatosis is fully expressed in females, with complete penetrance, in contrast to the manifestations of renal disease, which are in general more pronounced in men [7]. In the literature, all patients with the Alport Syndrome – Diffuse leiomyomatosis (ATS-DL) complex have been found to have deletions that encompass the 5' ends of the *COL4A5* and *COL4A6* genes and include the bidirectional promoter [5, 6, 8-17]. Unlike the *COL4A5* breakpoint, whose position varies among different patients, the *COL4A6* breakpoint is consistently found within intron II [5, 16]. Interestingly, larger deletions, extending beyond intron II of *COL4A6* do not cause DL and only result in ATS [13]. In this paper, we described three ATS-DL cases, two familial and one sporadic. MLPA analysis showed a deletion encompassing 5'-end of both *COL4A5* and *COL4A6* genes in the three cases. Array-CGH technique permitted a better definition of deletions' size. It confirmed that the proximal breakpoint did not extend beyond intron 2 in two cases and it showed a larger deletion extending beyond exon 3 in one case.

METHODS

Genomic DNA isolation

Genomic DNA from normal male 46,XY and normal female 46,XX was obtained from Promega. Genomic DNA of the patients was isolated from an EDTA peripheral blood sample by using a QIAamp DNA Blood Kit according to the manufacturer protocol (Qiagen, www.qiagen.com). The Hoechest dye binding assay was used on a DyNA Quant™ 200 Fluorometer (GE Healthcare) to determine the appropriate DNA concentration.

MLPA analysis

The MLPA analysis was performed using two commercially available MLPA kits, namely SALSA P191/P192 Alport kits (MRC-Holland, Amsterdam, Netherlands; http://www.mrc-holland.com). The assay consists of two reaction mixes containing probes for 48 of the 51 *COL4A5* exons. Probes for exons 8, 25 and 40 are not included. In addition, probes for COL4A6 exons 1, 1', and 2 are included. Details on probe sequences are available on the MRC-Holland web site (http://www.mrc-holland.com). This kit was previously tested on a series of patients with X-linked ATS and a patient with ATS-DL [18]. Briefly, 100 ng of genomic DNA was diluted with TE buffer to 5 µl, denatured at 98°C for 5 minutes and hybridized with SALSA Probe-mix at 60°C overnight. Ligase-65 mix was then added and ligation was performed at 54°C for 15 minutes. The ligase was successively inactivated by heat 98°C for 5 minutes. PCR reaction was performed in a 50 µl volume. Primers, dNTPs and polymerase were added and amplification was carried out for 35 cycles (30 seconds at 95°C, 30 seconds at 60°C and 60 seconds at 72 °C). The amplification products were separated on a ABI Prism 310 automatic sequencer and analyzed using the GenScan

software ver.3.1. For data analysis the values of peak sizes and areas were exported to an Excel table and compared with a normal control (MRC-Holland, Amsterdam, The Netherlands). A reduction in the ratio to about 0 in males and about 0.5 in females indicates a deletion in hemizygous and heterozygous form, respectively.

Array-CGH analysis

Oligo array-CGH analysis was performed to confirm MLPA results and to better define the size of the deletions. Array based CGH analysis was performed using two commercially available oligonucleotide microarrays containing respectively about 99.000 and 244.000 60-mer probes (Human Genome CGH Microarray 105A Kit, and 244A Agilent Technologies, Santa Clara, California) as previously reported [19]. The average spatial resolution of the 105A array is about 22Kb, for the 244K is about 9 Kb.

Real Time quantitative analysis

To evaluate the *COL4A6* gene dosage, we designed a Custom TaqMan Gene Expression Assay (Applied Biosystems, https://products.appliedbiosystems.com) specific for exon 3 of *COL4A6* gene (*COL4A6* exon 3 forward primer: 5'-GGGAGCTGTCAGTGTTTTCCT-3'; *COL4A6* exon 3 reverse primer: 5'-CCATGCCACTATTTGTCTTTCAACA-3'; *COL4A6* exon3 TaqMan probe: FAM 5'-ACTCTCGCTCCTTTCTC-3'). Quantitative PCR was carried out using an ABI prism 7000 (Applied Biosystems, Foster City California) in a 96-well optical plate with a final reaction volume of 50 µl. A total of 10 ng of DNA (10 µl) was dispensed in each of the four sample wells for triplicate reactions. Thermal cycling conditions included a pre-run of 2 min at 50°C and 10 min at 95°C. Cycle conditions were 40 cycles at 95°C for 15 sec and 60°C for 1 min, according to the TaqMan Universal PCR Protocol (PE Applied Biosystems, Foster City, CA, USA). The TaqMan Universal PCR Master Mix and Microamp reaction tubes were supplied by Applied Biosystems. The starting copy number of the unknown samples was determined using the comparative Ct method, as previously described [20].

CLINICAL DESCRIPTIONS

We describe two familial and one sporadic ATS-DL cases (Figure 1).

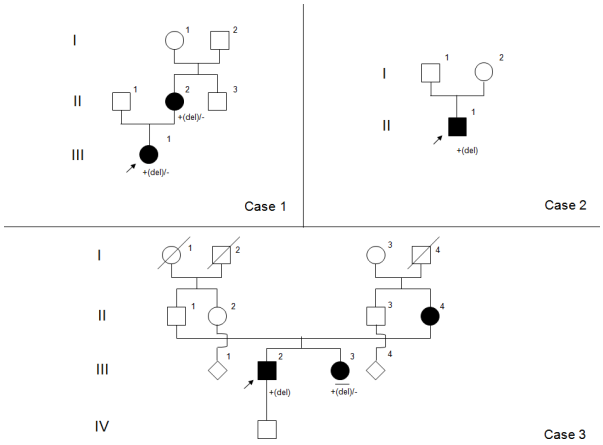

Figure 1. Pedigree of families. The figure represents the pedigree of cases 1-3. Symbol □= males, symbol ◯= females. Filled black symbols indicate individuals with a clinical diagnosis of ATS-DL. White symbols indicate individuals without clinical signs of the disease. An oblique bar indicates a deceased individual. The arrows indicate index patients. The genotype at *COL4A5-COL4A6* locus is indicated below each symbol as follows: - = wild type allele; + = mutated allele. Mutation type is indicated in brackets as del = deletion of *COL4A5-COL4A6* genes.

Case 1 (DAM)

We described a proband and her mother with a history of nephropathy and leiomyomatosis (Figure 1). The 9-year-old female proband (III-1) presented micro- and macrohematuria, proteinuria and a normal serum creatinine. The ultrastructural examination of a kidney biopsy performed at the age of 8 years revealed thickenings and lamellations of the GBM, compatible with a diagnosis of ATS. The audiological and ophtalmological examination resulted normal. She underwent anterior gastric hemifundoplication for esophageal achalasia with megaesophagus at 5 years of age, and subsequently she received a diagnosis of esophageal leiomyomatosis. The 39-year-old mother (II-2) presented microhaematuria, intermittent proteinuria and gross haematuric episodes since childhood. A diagnosis of esophageal achalasia with moderate esophagitis was made at 22 years of age, through esophagogastroscopy performed for dysphagia. She presented also uterine leiomyomatosis surgically treated at 25 years of age. Urinary and blood analysis revealed microhaematuria, proteinuria and normal serum creatinine.

Case 2 (PIN)

The patient is a 20 year-old male (II-1) with an unremarkable family history (Figure 1). At age three, because of swallowing difficulties and post-prandial vomiting, he was diagnosed with esophageal achalasia. At age four, he was diagnosed with microhaematuria. Routine auditory testing performed at age 6 showed initial sensorineural hearing loss, which progressively worsened and required hearing aids since age 11. Diffuse esophageal leiomyomatosis was diagnosed at age 9. At age 19 he had microhaematuria, proteinuria and moderately elevated creatinine levels.

Case 3 (RUG)

We described a two-generation family with a history of nephropathy and leiomyomatosis (Figure 1). The proband (III-2) is a 33-year-old male. The patient developed bilateral cataract and mixed hearing loss, which progressively worsened and required hearing aids since the age of 11. He has experienced dysphagia since childhood and he underwent resection of a histologically proven esophageal leiomyoma at 14 years. On this occasion, urinary analysis revealed microhaematuria and proteinuria. The ultrastructural analysis on a kidney-biopsy performed at 32 years displayed irregular thickness of the GBM with lamellations and basket weaving lesions, compatible with a diagnosis of ATS. The immunohistological analysis of renal distribution of type IV collagen chains showed absent α3(IV) and α5(IV) expression and a normal α1(IV) expression, compatible with X-linked ATS. At the time of examination, urinary and blood analysis revealed microhaematuria, proteinuria, normal creatinine clearance and serum creatinine. The 30 year-old sister (III-3) presented dysphagia since childhood. At 9 years of age barium swallow revealed a grossly dilated and floppy esophagus with abnormal peristalsis. A diagnosis of achalasia was then suspected. Following further analysis, a distal esophageal leiomyoma was diagnosed and it was surgically removed. She also reported constipation since childhood. A rectosigmoidal endoscopy at 21 years resulted compatible with rectal aganglionosis and

an histological examination of rectal biopsy at 24 years showed absence of the autonomic nervous system elements, confirming the diagnosis of Hirschprung disease. The gynaecologic examination revealed labia majora hyperthrophy with subcutaneous cysts. She also presented rectal and vaginal prolapse. At the time of genetic counselling, urinary and blood analysis revealed microhaematuria, proteinuria and a normal serum creatinine. She did not refer hearing deficit, but she has never performed auditory testing. Ocular examination resulted normal, except for the presence of mild myopia. The 57-year-old mother (II-2) referred achalasia surgically treated at the age of 9 years and hysterectomy for uterine leiomyomas at the age of 50 years. Urinary and blood analysis revealed microhaematuria, proteinuria and a mild increase of serum creatinine. She did not complain of hearing loss or ocular anomalies, but she has never performed auditory testing or an ophthalmologic examination.

MLPA analysis

An MLPA analysis was used to ascertain the presence of a *COL4A5-COL4A6* deletion in the three ATS-DL cases. In case 3, the proband was found to bear a deletion of *COL4A5* exon 1 and *COL4A6* exons 1, 1' and 2 (Figure 2). The deletion was found in heterozygous state in his sister (Figure 2). The same MLPA result was obtained for the proband and her mother of case 1 (data not shown). In case 2, a deletion of *COL4A5* exons 1-36 and *COL4A6* exons 1, 1', 2 was identified (data not shown).

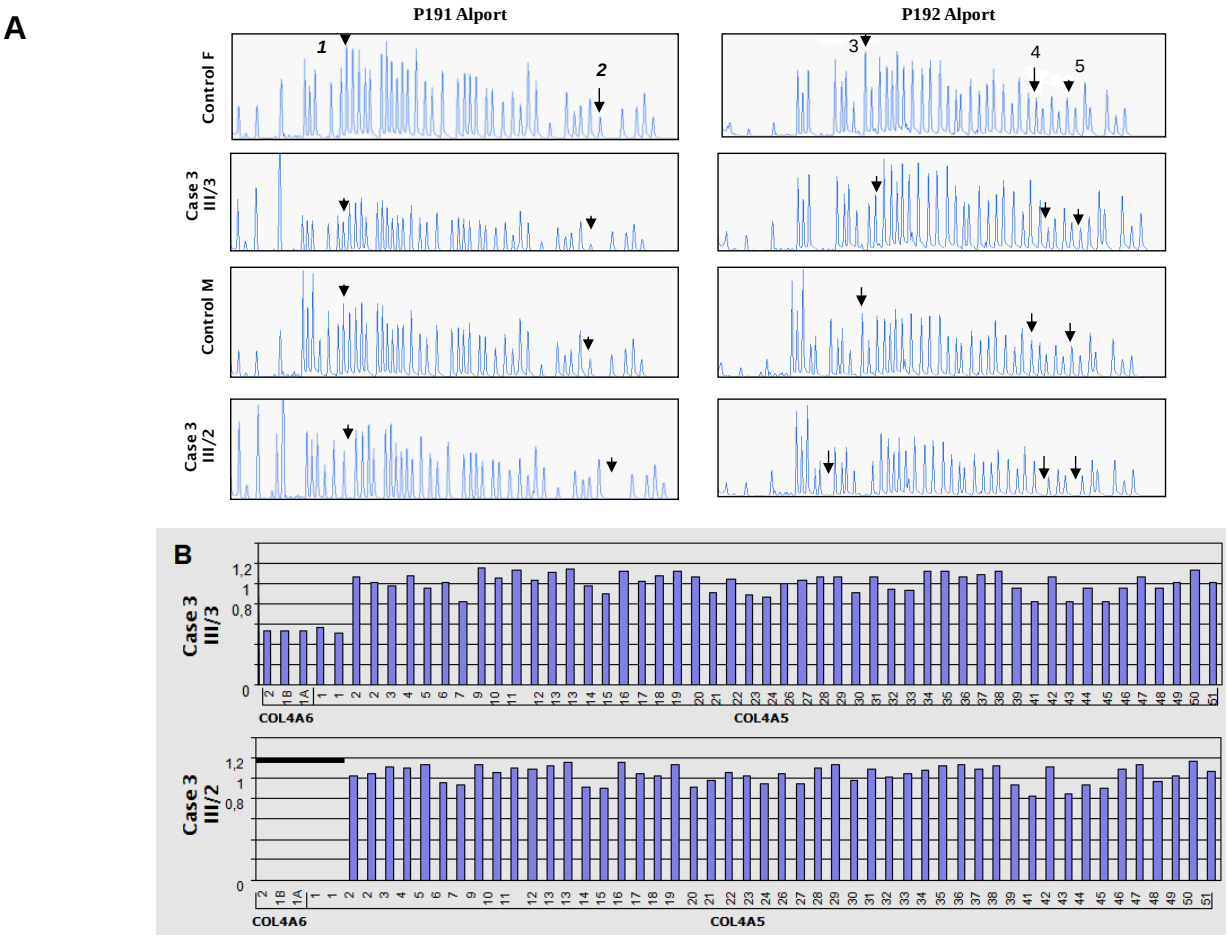

Figure 2. MLPA analysis. MLPA analysis results in case 3 showing the deletion in heterozygous (III-3) and hemizygous state (III-2). A) Electropherograms obtained with SALSA P191 Alport kit (on the left) and SALSA P192 Alport kit (on the right) for a normal control sample, proband (III-3) and sister (III-2) of case 3. Numbers and arrows indicate exon probes with reduced fluorescence signals respect to control samples. Numbers in the upper panels indicate specific MLPA probes: 1 = probe in *COL4A5* exon 1, 2 = *COL4A6* exon 1B, 3 = *COL4A5* exon 1, 4 = *COL4A5* exon 1A and 5 = *COL4A6* exon 1B. B) Peak area histograms for the two patients normalized with control samples. Exon dosage is reported on the y axis (normal values span from 0.8 to 1.2). MLPA analysis shows reduced peak area for the exons from *COL4A6* exon 2 to *COL4A5* exon 1, compatible with a heterozygous deletion in patient III-2 and with a hemizygous deletion in patient III-3. Deletions are indicated with heavy black lines.

Array-CGH analysis

Oligonucleotide array-CGH with an average spatial resolution of approximately 22 Kb (data not shown) and 9 kb (Figure 3) was performed in order to better define deletions' breakpoints. The analysis of ratio profiles revealed for case 1 and case 2 an interstitial deletion on the long arm of chromosome X (Figure 3). Based on the array findings, the deleted region identified in case 1 consists approximately 195 kb [46,XX del X(q22.3-22.3)]. The proximal breakpoint is mapped in Xq22.3 in *COL4A6* intron 3 (last oligonucleotide present located in 107.414 Mb, first deleted in 107.419 Mb position), while the distal breakpoint is located between 107.605 Mb and 107.619 Mb in Xq22.3 (last oligonucleotide deleted and first present, respectively) (Figure 3 and 4). The deleted region of case 2 is about 315 Kb in size (46, XX del (X)(q22.3;q22.3). The proximal breakpoint is mapped in Xq22.3 in *COL4A6* intron 2 (last oligonucleotide present located in 107.442 Mb, first deleted in 107,448 Mb position), while the distal breakpoint is located between 107,756 Mb and 107,764 Mb in Xq22.3 (last oligonucleotide deleted and first present, respectively). The array-CGH analysis of case 3 resulted in a ratio shifted to

the left for only one probe, localized in *COL4A5* intron 1 (107570801 Mb), indicating a deletion in that region (Figure 3 and 4). This result shows also that this deletion does not extend beyond *COL4A6* intron 2 and *COL4A5* intron 1 (Figure 3 and 4).

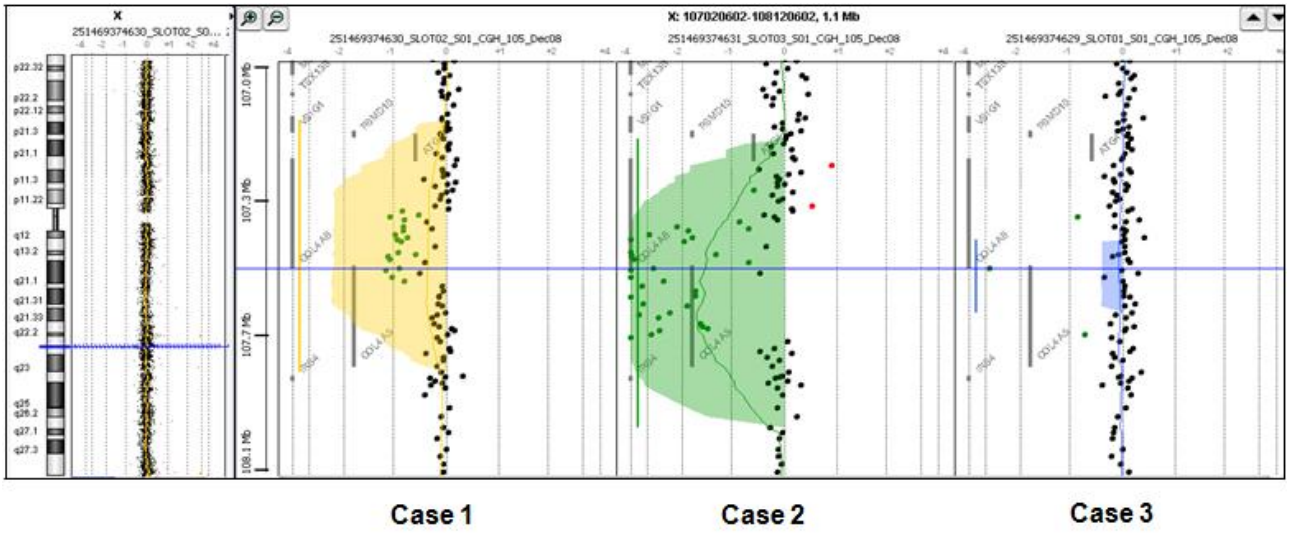

Figure 3. Array-CGH analysis. Array CGH 244K ratio profile. On the left, the X chromosome ideogram. On the right, the log2 ratio of chromosome X probes plotted as a function of chromosomal position. Oligos with a value of zero represent equal fluorescence intensity ratio between sample and reference DNAs. Each dot represents a single probe (oligo) spotted on the array. Copy number losses shift the ratio to the left (case 1 on the left; case 2 in the middle and case 3 on the right).

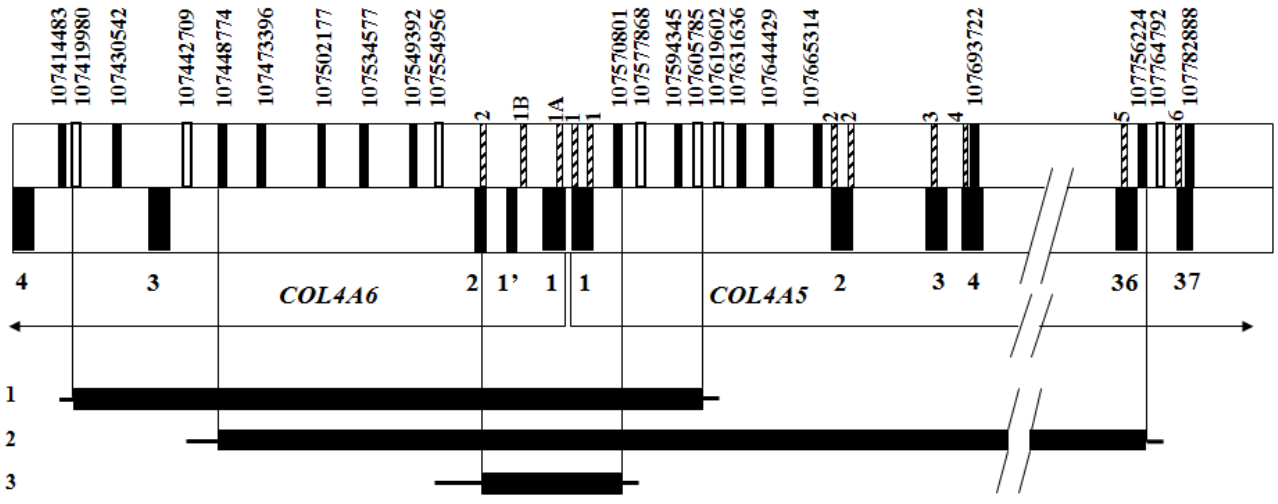

Figure 4. Deletions of the three ATS-DL cases. In the first bar, MLPA (zig zag lines) and Array-CGH probes (in black 105K probes and in white 244 K probes) of the region of interest are indicated. For Array-CGH probes, the exact genomic position is indicated (in the upper part). In the second bar, *COL4A5* and *COL4A6* exons of interest are indicated. At the bottom, black bars indicate the deletion extension of each case. The image is not on scale.

Real Time qPCR analysis.

In order to confirm the array-CGH results on the proximal breakpoint of case 1, a qPCR analysis by Real Time was performed using a probe located in exon 3 of COL4A6. The analysis demonstrated the presence of a deletion in patient II-2 of case 1, confirming previous results (Figure 5).

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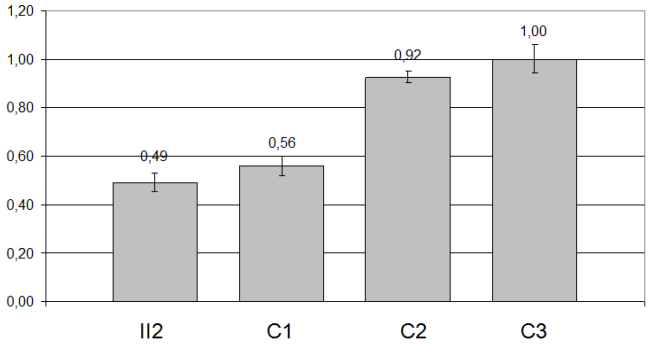

Figure 5. Real-time quantitative PCR validation experiment in exon 3 of *COL4A6* gene of case 1. *COL4A6* ddCT ratios (indicated in the Y-axis) and standard deviations of the patient (II2), of one male control sample (C1) and of two female control samples (C2 and C3). Compared to female controls, sample II2 shows ddCT ratio values of about 0.5, indicating a *COL4A6* deletion, as for control male.

DISCUSSION

In this paper we report on the application of MLPA and Array-CGH techniques to improve the definition of the *COL4A5-COL4A6* deletions in three ATS-DL cases. We report three cases, two familial and one sporadic, with a clinical diagnosis of ATS-DL. Family 3 has an interesting history. The male proband presented ultrastructural features typical of ATS but a mild form of nephropathy, in fact at the age of 33 years he presented a normal renal function. Even if the nephropathy in males with ATS-DL is usually severe, men with a mild renal involvement have been previously reported [14]. The clinical history of this patient stresses the fact that renal function and urinary status should be monitored in any patient with oesophageal leiomyomatosis. This is even more important in females, who usually show a milder renal involvement presenting isolated microhaematuria and who are at high risk of severe nephropathy in their male offspring. Furthermore, in almost all our cases the diagnosis of esophageal leiomyomatosis has been achieved after several years from a first diagnosis of esophageal achalasia and in family 3 the diagnosis of achalasia in proband's mother could likely represent a misdiagnosis of oesophageal leiomyomatosis, as previously described in other cases [6, 8, 21]. Given the clinical history of our patients, the possibility of ATS-DL should be considered in all ATS patients with dysphagia and/or a first diagnosis of esophageal achalasia. Even though in case 3, proband's sister had a definite diagnosis of Hirschsprung disease by histological examination, it has to be taken into account the possible misdiagnosis of Hirschsprung disease due to the presence of perirectal leiomyomatosis [6]. In order to identify the deletions in our ATS-DL patients, MLPA analysis has been performed. As expected, a deletion encompassing 5'-end of both *COL4A5* and *COL4A6* genes was found in the three cases: two deletions (cases 1 and 3) extending from *COL4A5* exon 1 to *COL4A6* exon 2 and one (case 2) from *COL4A5* exon 36 to *COL4A6* exon 2. According to the literature data, the proximal breakpoint of the deletion associated with ATS-DL is localized within the huge *COL4A6*

intron 2, about 127 kb in size (according to UCSC Genome Bioinformatics Site Human Mar. 2006 - NCBI36/hg18 – assembly; http://genome.ucsc.edu/), but the localization is variable [5, 13, 16]. Intron 2 is known to contain LINE1 repetitive elements which have been postulated to mediate deletion occurrence [15]. On the other end, larger deletions extending beyond intron 2 are known to result only in ATS [5, 13, 22, 23]. In order to better define deletions breakpoints, a 9 kb resolution array-CGH analysis has been performed. The array-CGH analysis confirmed in two cases a classical *COL4A6*/*COL4A5* deletion, with the 5' boundary located in *COL4A6* intron 2 (at a distance from *COL4A6* exon 2 of nearly 122 Kb in case 2 and 7 Kb in case 3). Surprisingly, in case 1 the deletion extended proximally beyond *COL4A6* exon 3 at a distance from *COL4A6* exon 3 of nearly 20 Kb (Fig. 4). These results were also confirmed by Real Time qPCR analysis (Figure 5). These findings are in contrast with literature data reporting deletions extending beyond COL4A6 exon 3 associated with isolated ATS [13]. Explanations for this discrepancy could be a possible lack of penetrance or later onset of leiomyomatosis in patients already reported. Till now, different hypotheses have been proposed to explain the correlation between ATS-DL phenotype and deletion extension. Mechanisms of smooth muscle overgrowth in ATS-DL are unknown and cannot be explained simply by the loss of the α5(IV) and α6(IV) chains. It has been postulated that the *COL4A6* intron II contains a gene and that a deletion with breakpoints within intron II could give rise to smooth-muscle tumors by gain of function, in a manner abrogated by more extensive deletions [8, 13, 15, 16, 24]. It has been also hypothesized that the partial deletion of *COL4A6* might cause a rearrangement of the gene eventually leading to overexpression of an alternative transcript in an inappropriate tissue leading to tumor development [13]. Another interesting theory supposes that the region extending from intergenic region to intron II influences the expression of neighbouring genes thorough a modification of chromatin structure [5]. This region could act as transcriptional regulatory element known as "Insulator", that modulate transcription by organizing the chromatin fiber within the nucleus through the establishment of higher-order domains of chromatin structure [25]. Overall our case one leads to reconsider the candidate region for the pathogenesis of the smooth muscle overgrowth in ATS-DL which seems to include also exon 3 beside intron 2 and the mechanisms that may be involved in the development of diffuse leyomiomatosis**.** In conclusion, we report on three extensively clinically characterized ATS-DL cases with a partial deletion of *COL4A5* and *COL4A6* genes. One of these three cases bears the largest deletion reported till now in the literature and it demonstrates that deletions extending beyond exon 3 of *COL4A6* are indeed associated with ATS-DL. Our data indicate that the MLPA analysis is a low cost, easy to use and reliable technique for the screening of patients with a clinical hypothesis of ATS-DL. However, MLPA analysis should include also *COL4A6* exon 3 and it has to be associated to other techniques in order to better define deletions breakpoints.

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2.4 A family with both dominant and recessive inheritance

I identified one mutation in the *COL4A3* gene in an interesting ATS family (STI) where both dominant and recessive inheritance is probably present in the same family.

Clinical description of family STI.

STI-III5- 39 year-old man, microhematuria and proteinuria since 10 years. He began to suffer from a nephritic syndrome at 24 years and he developed end-stage renal disease at 31 years. He underwent renal transplantation at 32 years.

STI-III4- 41 year-old female, microematuria and episodes of macroematuria since 4 years, proteinuria since 11 years and renal failure since 18 years. She developed end-stage renal disease at 19 years and she underwent renal transplantation at 21 years. Electron microscopy of renal biopsy suggested an ATS diagnosis.

STI-III3- 42 year-old man, microematuria and proteinuria since 10 years and renal failure since 21 years and he developed end-stage renal disease at 26 years. He underwent renal transplantation at 29 years.

STI-III1- 44 year-old man, microscopic hematuria and proteinuria since 38 years.

STI-II1 and **STI-II4**- They both did not show renal pathologic signs.

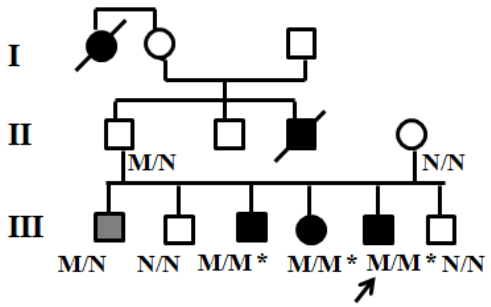

Figure 4. Pedigree of family. Open squares are males and open circles are females. Filled grey symbols are individuals with microhemauturia and proteinuria. Filled black symbols indicate individuals with microhematuria plus renal failure. White symbols indicate individuals without clinical sings of the disease. The arrows indicate the index patients. The genotype at *COL4A3* locus is indicated below each symbol as follows: N=wild type allele; M=mutated allele; M*=allele with a second hypothetical mutation.

Molecular analysis.

DHPLC analysis and subsequent direct sequencing of exon 37 of the *COL4A3* gene resulted in the identification of a mutation, that caused a glycine substitution in the collagenous domain of the protein (p.G1045V, c.3134G>T) (Figure 5). The mutation was present in all the affected family members and in the apparently healthy father (Figure 4).

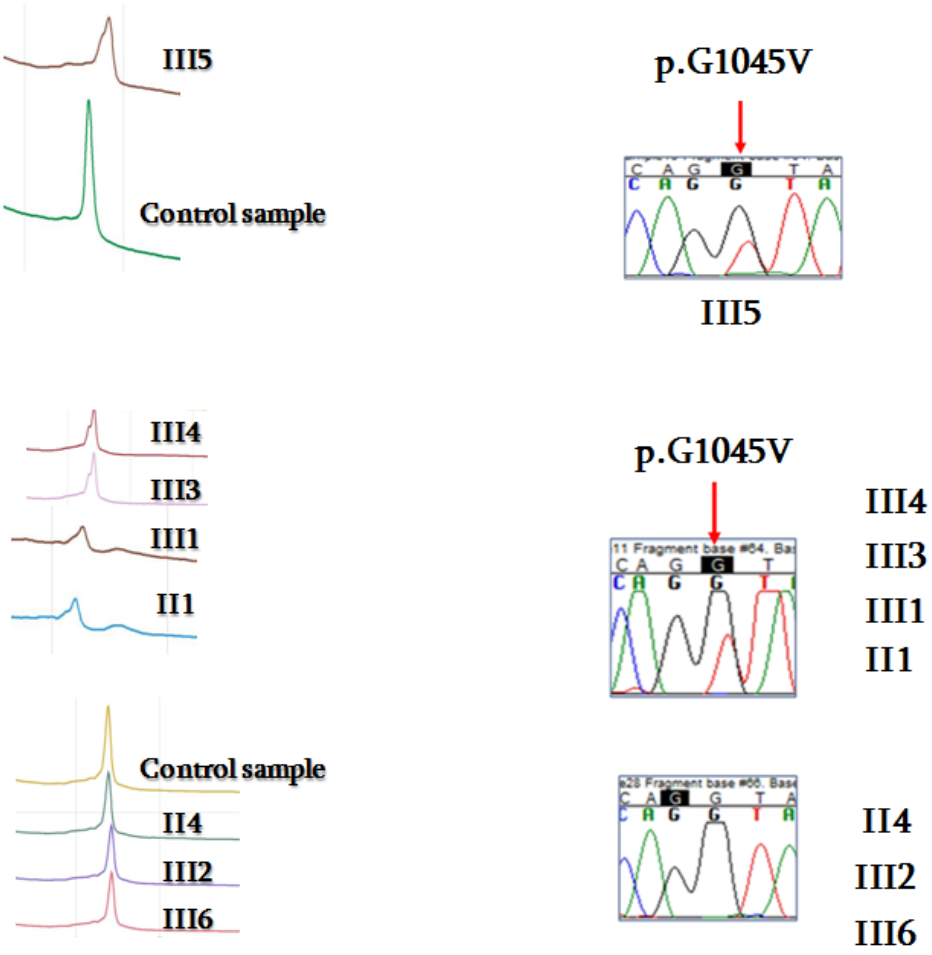

Figure 5 . DHPLC pattern and sequence of identified mutation. The left side of each panel represents the DHPLC pattern of proband (III5) and of relatives (II1, II4, III1, III2, III3, III4, III6). On the right side of each panel the mutated sequence is reported with the mutation written above. Note that the control samples and relatives (II4, III2, III6) show a unique peak (homoduplex peak) while proband (III5) and relatives (II1, III1, III3, III4) show a heteroduplex peak.

3. DISCUSSION and FUTURE PERSPECTIVES

3. DISCUSSION and FUTURE PERSPECTIVES

The ATS is considered one of the most common inherited glomerulonephritis often associated with deafness and ocular lesions. While the X-linked and the autosomal recessive forms are well known, the autosomal dominant form is not well acknowledged. In addition, intra-familial phenotype variability is reported and the progression of renal damage does not strictly correlate with the kind of mutation. In fact, this observation suggests that other factors, beside *COL4A4* and *COL4A3* mutation, may influence the clinical outcome. These factors may or may not be of genetic nature (lifestyle, diet). *COL4A4* and *COL4A3* genes contain several polymorphisms.[20] It could be interesting to test whether some of them are functional variants and whether they are responsible for part of the phenotypic variability. In addition, functional variants in other proteins that are key player in renal filtration may act as modifiers. Patients with more or less proteinuria may have more or less functioning variants of these proteins.

In my PhD study, by the analysis of my cohort of ATS patients, I contributed to clarify the autosomal ATS pathogenesis, firstly by the analysis of families with ATS-AR reported *by Longo I, et al: Nephrol Dial Transplant 2006* and finally by the analysis of 8 families with ATS-AD. Recently, I identified one mutation in the COL4A3 gene in an interesting ATS family (STI) where both dominant and recessive inheritance is present. In this family an initial clinical analysis of the proband, of the brother and the sister showed that they developed end-stage renal disease at the age of 31, 26 and 19 years respectively, suggesting the hypothesis of a recessive ATS. This hypothesis is confirmed by the fact that the proband's father presents a mutation with a normal clinical profile. However, the older brother, aged 44, showed only microscopic hematuria and proteinuria suggesting an autosomal dominant form. One of the brother is affected by a dominant form while the proband, the sister and the other brother are affected by a recessive form. This suggest a new model including both dominant and recessive inheritance, in same family.

The second mutation supporting the above reported model in the patient 2740 has not been found. One can guess that, using DHPLC technique, the presence of polymorphism in COL4A3 gene, may hide the presence of a pathogenic mutation.[10] Alternatively, an intronic mutation might have been lost since we analyse only the coding sequence and exon/intron junction. In addition, a large deletion on the other allele can not be excluded since MLPA analysis is not yet available for this gene. Finally, the second mutation could be in *COL4A4* gene, not yet analyzed, but up to now, a compound heterozygous state from two different autosomal ATS genes (*COL4A3* and *COL4A4*) has not been reported yet.

In the last year of my PhD I also contributed to the analysis of three families with ATS-DL. X-linked ATS is rarely associated with diffuse leiomyomatosis (ATS-DL), a benign hypertrophy of the visceral smooth muscle in gastrointestinal, respiratory and female reproductive tracts. All patients with the ATS-DL complex have been found to have deletions that encompass the 5' ends of the *COL4A5* and *COL4A6* genes and include the bidirectional promoter. Unlike the *COL4A5* breakpoint, whose position varies among different patients, the *COL4A6* breakpoint has been always invariably found within intron II.[7,8] Instead, my work has demonstrated that a deletion at *COL4A5-COL4A6* locus associated with ATS-DL can extend proximally beyond intron II of *COL4A6*. For the analysis of three families and in order to deeply define the deletions breakpoints a combination of array-CGH technology and quantitative Real Time PCR has been used. In particular these techniques are particularly of diagnostic relevance in cases where no male probands are available such as the case of family 1 (DAM) reported and they have substituted the previously employed Southern Blotting technique. With this technique, I have identified in a family the most proximal breakpoint never reported in ATS-DL patients. In fact, larger deletions, extending beyond intron II of *COL4A6*, usually do not cause DL and only result in ATS, but this family a deletion extending proximally beyond exon 3 of

COL4A6 and cause ATS-DL. This in the largest deletion reported to date associated with ATS-DL and this case should lead us to reconsider the mechanisms that may be involved in the development of diffuse leyomiomatosis (Result 2.3).

On clinical ground it is worth noting that the diagnosis of ATS is difficult since mutations in these large genes are often private and there are not mutational hot spots, with the exception of two cluster of families reported in Results 2.1, the majority are private mutations. A recent alternative strategy that may overcome the difficulty of the analysis of such a huge genes is the new technique "next-generation sequencing". This recent introduction of instruments capable of producing millions of DNA sequence reads in a single run is rapidly changing the landscape of genetics, providing the ability to answer questions with heretofore unimaginable speed. [26]

4. REFERENCES

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