Prognostic Value of Glomerular Collagen IV Immunofluorescence Studies in Male Patients with X-Linked Alport Syndrome

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Summary

Background and objectives X-linked Alport syndrome (X-AS) is caused by mutations of the *COL4A5* gene, which encodes for the collagen IV α5 chain (α5[COLIV]), resulting in structural and functional abnormalities of the glomerular basement membrane (GBM) and leading to CKD. The aim of the present study was to evaluate the prognostic value of residual collagen IV chain expression in the GBM of patients with X-AS.

Design, setting, participants, & measurements The medical records of 22 patients with X-AS from 21 unrelated families collected between 1987 and 2009 were reviewed (median age at last follow-up, 19.9 years; range, 5.4–35.1 years); GBM expression of α1, α3, and α5(COLIV) chains was assessed by immunofluorescence microscopy.

Results GBM distribution of the α5(COLIV) chain was diffuse in 1 and segmental or absent in 21 of the 22 patients; the expression of the α3(COLIV) chain was diffuse in 5 of 22 patients and segmental or absent in 17 of 22 patients. Patients with diffuse staining for the α3(COLIV) chain presented with proteinuria significantly later (median age, 16.9 versus 6.1 years; P=0.02) and reached an estimated GFR < 90 ml/min per 1.73 m^2 at an older age (median age, 27.0 versus 14.9 years; P=0.01) compared with patients with segmental or absent staining. Two thirds of patients with abnormal α3(COLIV) expression by immunofluorescence studies had null or truncating *COL4A5* mutations, as opposed to none of the 4 tested patients with diffuse α3(COLIV) chain glomerular distribution.

Conclusions These results indicate that maintained expression of the α3(COLIV) chain is an early positive prognostic marker in patients with X-linked Alport symdrome.

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Introduction

Alport syndrome (AS) is caused by genetic abnormalities of collagen IV α3, α4, or α5 chains (α3[COLIV], α4[COLIV], α5[COLIV]) that are constitutive components of the glomerular basement membrane (GBM) (1,2). Approximately 80% of cases demonstrate an X-linked inheritance (X-AS) and are caused by mutations in the *COL4A5* gene (3,4); 15%–20% of cases are transmitted with an autosomal-recessive mode and are caused by homozygous or compound heterozygous mutations in the *COL4A3* or *COL4A4* genes (5,6). Finally, a minority of cases are caused by specific mutations in the *COL4A3* or *COL4A4* genes and demonstrate an autosomal-dominant transmission (6, 7, 8, 9). The exact prevalence of autosomal-dominant AS is still unknown, but it could be higher than previously reported; recent technical advances in the molecular diagnosis of AS will probably help define the relative contribution of these forms (10).

From the renal standpoint, patients with AS develop a characteristic sequence of signs that begin with early-onset hematuria followed by proteinuria, decreased GFR, and, eventually, ESRD. Patients with more severe disease have earlier onset of proteinuria, progress more rapidly to ESRD, and more often have extrarenal symptoms, including sensory-neural deafness and ocular lesions (anterior lenticonus and perimacular flecks).

To date, few studies have attempted to correlate the clinical phenotype with the severity of GBM abnormalities by electron microscopy (EM) and with the GBM expression of collagen chains (11–13). In most X-AS cases, immunofluorescence (IF) studies show absent GBM expression of all constitutive collagen IV chains (α3, α4, and α5) (12,14). In a minority of patients, however, partial or normal expression can be observed (12,15), whereas female carriers often demonstrate a segmental pattern of expression by IF studies (12). Similarly to X-AS, patients with autosomal-recessive AS generally have no detectable α3, α4, and α5(COLIV) chains in their GBM; however, positive immunoreactivity for α5(COLIV) chain is observed in other basement membranes, including the renal

capsule, the renal tubular structures, and the epidermal basement membrane (EBM) (5). Data in autosomal-dominant AS are very limited.

In 2001, Barsotti and coworkers observed a negative correlation between the severity of GBM lesions by EM and the degree of glomerular expression of the α3(COLIV) chain by IF microscopy (11); they observed no significant correlation with the distribution of the α5(COLIV) chain (11). On the basis of these observations, we have speculated that the distribution of α3(COLIV) in the GBM of patients with X-AS could help predict clinical evolution. To this end, we used IF microscopy to retrospectively analyze the GBM expression of collagen IV chains in renal biopsy samples obtained from patients with X-AS, and we correlated results of these studies with the evolution of renal function and with the development of extrarenal symptoms.

Materials and Methods

Patients

All patients followed at the Bambino Gesù Children's Hospital for X-AS before age 18 years were included in this study. No patient had significant proteinuria at the first evaluation. IF microscopy was performed on renal biopsy samples obtained between 1987 and 2009 to assess the glomerular expression of α3(COLIV) and α5(COLIV) chains. All biopsies were performed at the Bambino Gesù Children's Hospital and demonstrated characteristic GBM changes by EM (Supplemental Table 1). The diagnosis of X-AS was substantiated in all cases by the demonstration of abnormal α5(COLIV) chain distribution in the EBM or by the identification of *COL4A5* gene mutations (Supplemental Table 1).

This study was approved by the ethical committee of the Bambino Gesù Children's Hospital, who also reviewed the results before submission of the manuscript; parents of underage patients or adult patients gave their informed consent to perform IF studies, in addition to renal biopsies, EBM studies, and genetic analyses, which were performed for diagnostic purposes.

Medical records were retrospectively analyzed. For each patient, the age of appearance of proteinuria (defined as a urinary protein-to-creatinine ratio > 0.5 mg/mg), the age at development of stage II CKD (i.e., estimated GFR [eGFR] < 90 ml/min per 1.73 m^2), and the age at which ESRD developed (defined by the date of initiation of dialysis or preemptive transplantation) were recorded. eGFR was calculated with the Schwartz equation (16). In addition, patient characteristics at the time of biopsy were collected, as well as extrarenal symptoms. Hearing impairment or deafness was defined as the need for hearing aids or by a hearing threshold >30 dB for frequencies >2000 Hz. Ocular abnormalities were defined by the presence of an anterior lenticonus by slit-lamp examination or of retinal macular flecks by ocular fundus examination. For all patients, clinical and biologic data were available at least twice a year. Hearing tests and eye examinations were generally performed yearly.

Mutation analyses were performed using standard genetic tests with techniques that varied over the years; part of these mutations and the methods used to detected them have been previously published (3,17–19); in a few patients given a diagnosis in more recent years, genetic testing was not performed because X-AS was diagnosed by the demonstration of negative α5(COLIV) expression in the EBM.

Immunohistochemistry

Monoclonal antibodies to α1(COLIV), α3(COLIV), and α5(COLIV) were purchased from Weislab AB (Lund, Sweden). Standard renal IF analyses were performed on frozen sections stored at −80°C according to the manufacturer's protocol. Briefly, 4-micron cryostate sections were fixed in acetone for 10 minutes and washed in PBS. Sections to be stained with anti–5 chain antibodies were denatured by incubation for 10 minutes in 6 mol/L urea − 0.1 mol/L glycine-HCl buffer (pH, 3.5) at 4°C to uncover the antigen and were washed in distilled water.

The glomerular distribution of the α1(COLIV) chain was used as internal control; nonimmune mouse sera were used as external control. Glomerular distribution of collagen chains was defined as diffuse, segmental, or absent, as illustrated in Figure 1. At least three sections were taken at different levels of the frozen specimen for all patients. All biopsy specimens were separately analyzed by two observers, who were blinded to the clinical evolution of patients. No difficulties emerged in labeling the pattern of collagen chain distribution, which was absent in the majority of samples (see the Results section); no disagreement emerged between the two observers.

Statistical Analyses

Survival data were analyzed by the Kaplan-Meyer distribution and were compared with the log-rank test. Outcomes included the age at development of proteinuria, CKD, ESRD, and deafness. No patients had significant proteinuria when they first presented, allowing us to establish the age at onset of proteinuria. Three patients had significant deafness when X-AS was diagnosed; for these patients, the age at diagnosis was used as the age of appearance of deafness. Differences between groups were assessed by the Mann-Whitney *U* test for continuous variables and with the Fisher exact test for dichotomous variables. All tests were two sided, and $P<0.05$ represented statistically significant differences.

Results

Twenty-two male patients from 21 unrelated families were included in the study. The median age at the time of biopsy was 9.3 years (range, 1.6–23.3 years). A positive family history was present in most cases. All patients had characteristic GBM lesions by EM and abnormal distribution of the α5(COLIV) chain in their EBM (Supplemental Table 1). The mean age at the last follow-up was 20.0 years (range, 5.4–35.1 years).

Proteinuria developed in 21 of 22 patients at a median age of 7.2 years (range, 1.9–23.2 years); 17 of 22 developed CKD or ESRD during follow-up. Deafness was present in 16 of 22 patients, and ocular lesions were observed in 2 of 22 (1 with anterior lenticonus and 1 with retinal flecks). The median age at the last follow-up was 20.4 years (range, 5.4–35.1 years).

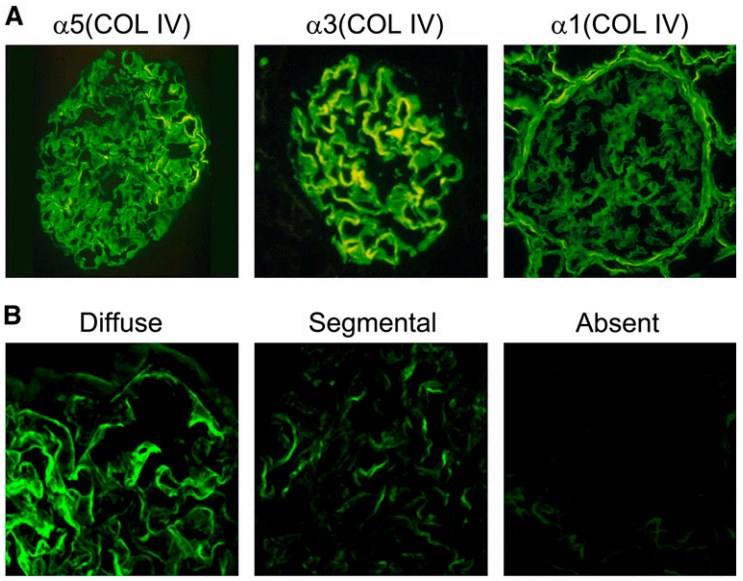

Figure 1. | **Immunohistochemistry of α5(COLIV) and α3(COLIV) in glomerular basement membrane.** (A) Normal glomerular distribution of α5(COLIV) and α3(COLIV); staining with α1(COLIV) chain (Bowman capsule and tubular basal membrane) was used as internal control. (B) Examples of diffuse, segmental, and absent staining for the α3(COLIV) chain in patients with X-linked Alport syndrome.

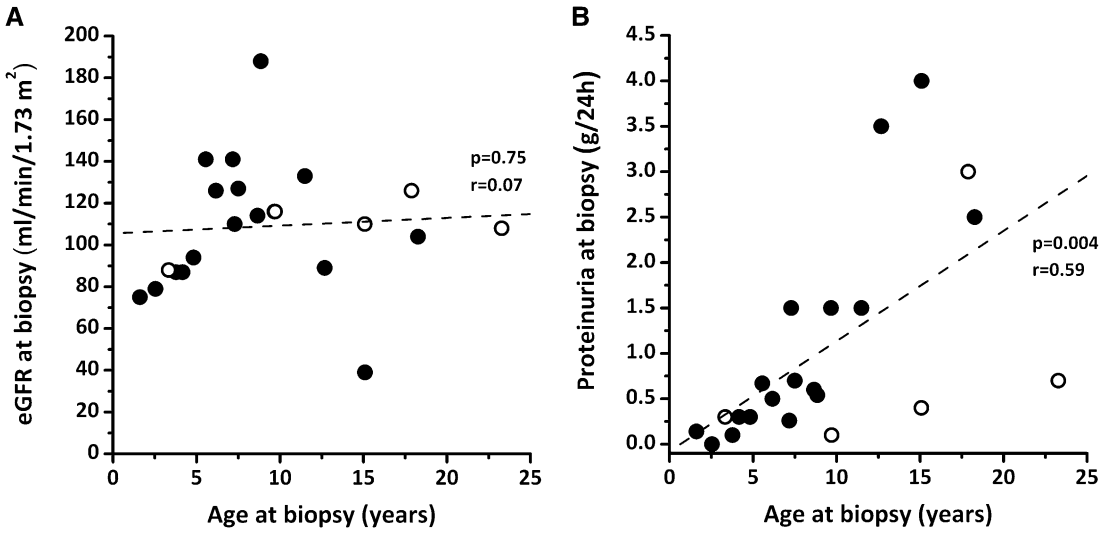

Figure 2. | **Renal measures at biopsy.** (A and B) Correlation between age at biopsy and estimated GFR (eGFR) (A) and between the age at biopsy and proteinuria (B). Empty circles indicate patients with diffuse α3 chain immunofluorescence pattern; filled circles indicate patients with segmental or absent α3 expression by immunofluorescence.

Renal biopsies were performed at different ages, usually upon development of significant proteinuria, when renal function was still normal or close to the normal range (Figure 2A). Patient age and eGFR at biopsy were not correlated; a positive correlation was observed between the age at biopsy and proteinuria at biopsy ($P<0.004$) (Figure 2B). Normal glomerular distribution of the α1(COLIV) chains was observed in all cases (data not shown). The distribution of the α5(COLIV) chain was diffuse in 1 patient, segmental in 2 patients, and absent in the remaining 19 patients. The distribution along the GBM of the α3 (COLIV) chain exhibited a diffuse pattern in 5 of 22 biopsy specimens; in the remaining 17 specimens, the observed pattern was abnormal (3 segmental and 14 absent).

The prevalence rates of ocular lesions and of hearing impairment, as well as patient characteristics at biopsy and number of criteria for AS diagnoses, were similar between patients with normal and abnormal glomerular α3(COLIV) chain expression by IF studies (Table 1).

The progression of renal disease was analyzed using the Kaplan-Meyer curves that are shown in Figure 3, A–D. Patients with normal renal distribution of the α3(COLIV)

Table 1. Patient characteristics

Characteristic	α3(COL IV) Staining in the GBM		P Value
	Diffuse (*n*=5)	Absent or Segmental (*n*=17)	
Patient characteristics at biopsy			
Age (yr)	15.1 (3.3–23.3)	7.3 (1.6–18.3)	0.12
Proteinuria (g/m^2 per 24 hr)	0.4 (0.1–1.7)	0.6 (0–2.7)	0.76
eGFR (ml/min per 1.73 m^2)	110 (88–126)	110 (39–188)	0.94
Criteria for diagnosis of Alport syndrome, *n* (%)			
Positive family history	4/5 (80)	16/17 (94)	0.41
Absent or segmental α5(COLIV) in GBM	4/5 (80)	17/17 (100)	0.23
COL4A5 mutation	4/5 (80)	12/13 (93)	0.49
Absent or segmental α5(COLIV) in EBM	5/5 (100)	17/17 (100)	>0.99
Patient characteristics at the last follow-up			
Age (yr)	24.9 (20.5–35.1)	18.3 (5.4–31.9)	0.51
CKD, *n/n* (%)	3/5 (60)	14/17 (82)	0.55
ESRD, *n /n* (%)	1/5 (20)	11/17 (65)	0.14
Proteinuria, *n/n* (%)	4/5 (80)	17/17 (100)	0.23
Deafness, *n/n* (%)	4/5 (80)	12/17 (71)	>0.99

Values with ranges are medians. GBM, glomerular basement membrane; eGFR, estimated GFR; EBM, epidermal basement membrane.

chain had more delayed appearance of proteinuria (median age, 16.9 versus 6.1 years; *P*=0.02) (Figure 3A) and developed CKD (eGFR < 90 ml/min per 1.73 m^2) at an older age (median age, 27.0 versus 14.9 years; *P*=0.01) (Figure 3C), as well as ESRD (*P*=0.04) (Figure 3D) and deafness (median age, 27.0 versus 12.3 years; *P*=0.03) (Figure 3B), compared with patients with abnormal (segmental or absent) expression of the α3(COLIV) chain by IF microscopy.

Genetic analyses were performed in 17 families (18 patients); mutations in the *COL4A5* gene were detected in 15 families. As illustrated in Table 2, 4 of 12 patients with abnormal α3(COLIV) chain distribution had mutations that severely disrupt the α5(COLIV) amino acid sequence (truncating or null mutations), whereas all tested patients with a diffuse pattern of expression of the α3 (COLIV) chain had in-frame deletions, missense mutations, or splice site mutations.

Discussion

The clinical evolution of X-AS is known to vary significantly between patients of different families (4). This is due, at least in part, to differences in the severity of *COL4A5* mutations, as indicated by the demonstration of genotype-phenotype correlations (albeit less robust than correlations in other renal diseases) (3,4). More specifically, associations between the type of mutation and the clinical evolution were observed after patients were grouped per category of mutation (3,4,20,21).

The typical sequence of GBM changes in patients with X-AS syndrome has been well documented and reproduced in animal models (22); to date, however, correlation data between COLIV chain expression and clinical evolution are lacking.

IF microscopy studies in renal biopsy specimens obtained from patients with X-AS have shown a strong inverse correlation between α3(COLIV) glomerular staining and the severity of ultrastructural GBM lesions (11). These studies suggest that in X-AS, preservation of the assembling processes that are required for the formation of type IV collagen trimers delays the development of structural GBM changes.

In humans, mutations in any of the three type IV collagen chains frequently inhibit the expression of the other two chains in the GBM (14). Likewise, *col4a3* knockout mice express neither α4 nor α5(COLIV) chains in their glomeruli (23,24). In this respect, the α3 NC1 domain appears to be particularly important for the assembly of type IV collagen protomers (22). If the α3 NC1 sequence is replaced by a human α5 NC1 sequence, the glomerular expression of both α4 and α5 chains is severely compromised, resulting in glomerular lesions that are indistinguishable from those observed in knockout animals that do not express the α3 (COLIV) protein (25).

This study shows that mutations that preserve the expression of the α3(COLIV) chain in the GBM, as detected by IF microscopy, are associated with less severe disease. Although negative glomerular staining for the α3 chain may result from protein misfolding altering or hiding the epitope recognized by the antibody, current evidence suggests that the α3 chain is most likely not expressed as a consequence of *COL4A5* mutations that produce posttranslational defects precluding the assembly of monomers, which are rapidly degraded and are no longer detectable by immunohistochemistry (14,26).

Unlike "simpler" fibrillar networks, such as type I collagen, proteins that compose type IV collagen are organized in complex quaternary structures that require sophisticated assembly processes (14,26); in particular, current data show that α3 chains can be incorporated into the GBM only as α3-α4-α5 trimers (26). Therefore, negative α5 chain staining in renal biopsy specimens from patients with preserved α3 distribution most likely indicates that IF microscopy using anti-α3 antibodies is more sensitive in detecting collagen IV heterotrimers in the GBM of patients with X-AS. In this view, patients with less severe disease have *COL4A5* mutations that do

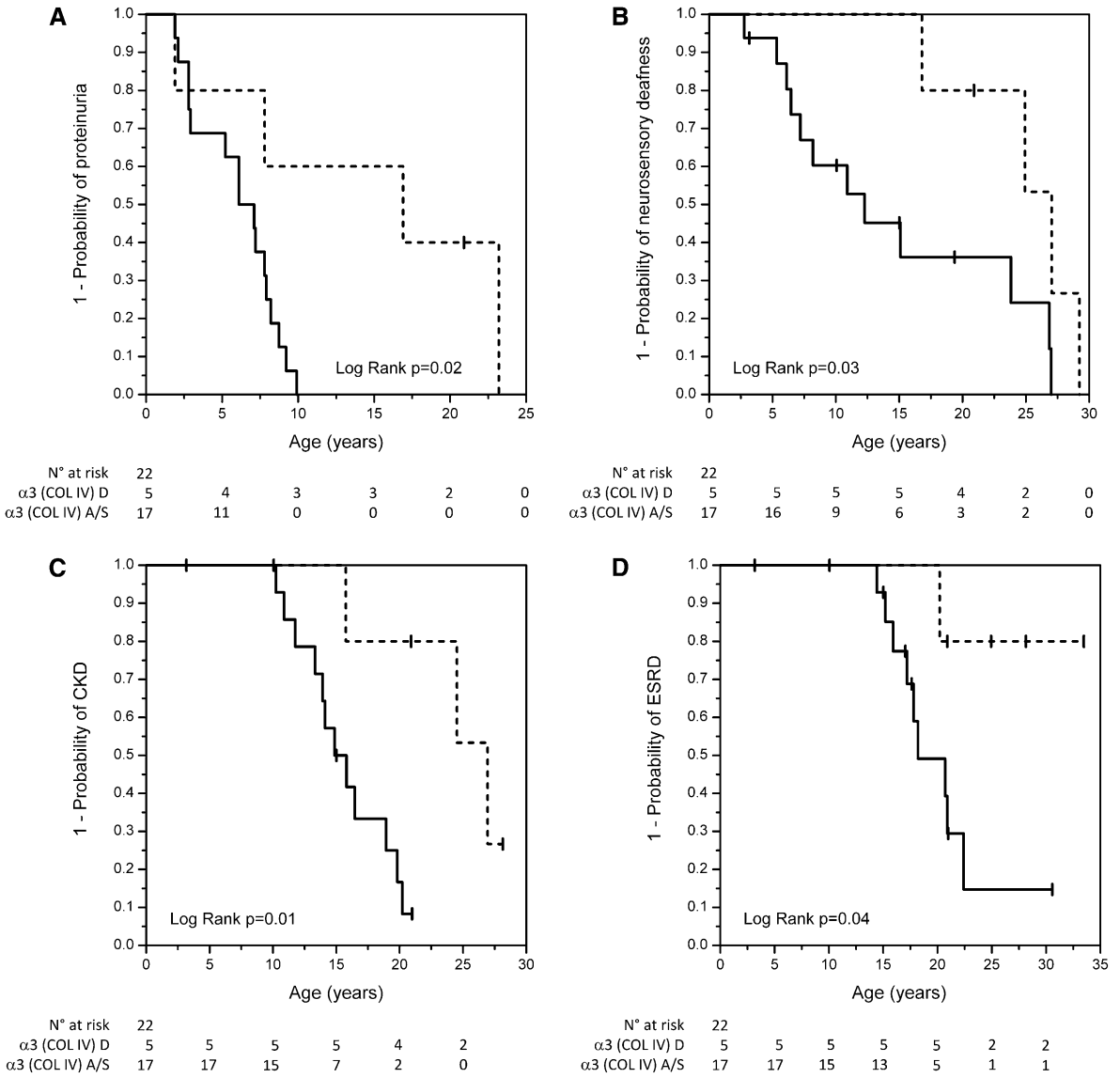

Figure 3. | Survival curves in patients with or without diffuse α3(COLIV) chain expression. Probability of developing proteinuria (A), sensorineural deafness (B), CKD (C), and ESRD (D) according to the immunofluorescence staining of the α3(COLIV) chain in the glomerular basement membrane. CKD was defined as stage II or greater. Dashed lines indicate patients with diffuse α3 chain expression by immunofluorescence; solid lines indicate patients with segmental or absent α3 staining. Vertical bars indicate censured data. A, absent; D, diffuse; S, segmental.

not hamper the assembly of collagen IV protomers. Negative α5 chain immunostaining in these patients may result from *COL4A5* mutations that directly alter the amino acid sequence of the epitope or that cause misfolding of the protein hiding or altering the epitope recognized by the antibodies. More studies are needed to reach a final interpretation of the observed discrepancies between α3 and α5 IF microscopy results, as well as to analyze in detail the possibility that some staining, although unlikely, may correspond to proteins retained inside cells; the immunofluorescence technique used in this study did not allow us to perform these analyses, even at high magnification. Of note, lack of correlation between α5(COLIV) chain expression by IF microscopy and ultrastructural GBM changes has been previously reported (11).

Although highly significant, our results are limited by the number of patients with normal glomerular distribution of the α3 chain. The observed prevalence of these patients in our cohort may not be representative of the entire X-AS population and may in part reflect a selection bias because all enrolled patients were recruited in a pediatric institution; patients with preserved glomerular distribution of the α3(COLIV) chain may be more prevalent in adult cohorts that are more likely to include patients with late-onset proteinuria.

Arguably, the immunohistochemical detection of collagen IV chains in the GBM of patients with X-AS may decrease during progression of their renal disease, which may have influenced results of this study. Most patients, however, underwent a renal biopsy when they developed

Table 2. *COL4A5* mutations and results of glomerular immunofluorescence studies

Patient No.	GBM α3	GBM α5	Position	*COL4A5* Mutation		Type of Mutation	
				Amino Acid	Nucleotide	Missense Splice site In-frame deletion	Truncating Null
1	D	S	Exon 17	p.Gly325Glu	c.1176G>A	X	
2	D	A	Exon 34	p.Gly988Glu	c.3165G>A	X	
3	D	A	Intron 14	—	c.834+2T>C	X	
4	D	A	Intron 14	—	c.834+2T>C	X	
5	D	A	Nondetected				
6	S	A	Exon 30	p.Gly828Arg	c.2684G>C	X	
7	S	S	Exon 11	p.Pro212del	c.836_838delCCA	X	
8	S	A	Nondetected				
9	A	A	Large deletion				X
10	A	A	Exon 37	p.Gly1107Arg	c.3521G >C	X	
11	A	A	Exon 24	p.Gly545Val	c.1836G>T	X	
12	A	A	Exon 29	p.Gly781×	c.2543G>T		X
13	A	A	Exon 27	p.Pro686fsX735	c.2259 delC		X
14	A	A	Complex rearrangement				X
15	A	A	Exon 31	p.Gly869Arg	c.2799G>A		X
16	A	A	Exon 37	p.gly1113fsX1151	c.3539_3540insCCGT		X
17	A	A	Exon 19	p.Arg373×	c.1319C>T		X
18	A	A	Exon 34	p.Ile974fs1009	c.3124insC		X

Mutations were not identified in patients 5 and 8 and were not studied in patients 19–22. The α3(COLIV) chain distribution was diffuse in patient 5, segmental in patient 8, and absent in the remaining patients; all had negative α5(COLIV) chain staining. GBM, glomerular basement membrane; D, diffuse; S, segmental; A, absent.

significant proteinuria; therefore, patients with altered α3 (COLIV) distribution had more severe disease and were more likely to undergo biopsy earlier, although differences did not reach statistical significance. This makes the results of our IF studies unlikely to have been influenced by the timing of the renal biopsy.

Finally, correlation between the severity of renal involvement and extrarenal symptoms is well established in X-AS (3,4,20,27). Likewise, we also observed that patients with early-onset CKD developed deafness earlier. Ocular abnormalities were detected only in two patients, in clear contrast with data from the literature; this may reflect in part the high prevalence of young patients in our cohort and in part failure to diagnose subtle ocular lesions.

In conclusion, this study indicates that maintained expression of the α3(COLIV) chain in renal biopsy specimens of patients with X-AS represents an early positive prognostic factor. Lack of detection of the α3 chain is likely to be a consequence of *COL4A5* mutations that prevent the assembly of α3-α4-α5 trimers; these data may therefore be relevant for deciphering genotype-phenotype correlations in X-AS. Assessing the glomerular expression of the α3(COLIV) chain by IF microscopy may represent a useful tool for testing new therapies in this disease.

Disclosures

None.

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