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Negative Staining for COL4A5 Correlates With Worse Prognosis and More Severe Ultrastructural Alterations in Males With Alport Syndrome

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Introduction: Alport syndrome (AS) is a genetic disorder characterized by progressive hematuric nephropathy with or without sensorineural hearing loss and ocular lesions. Previous studies on AS included mostly children.

Methods: To determine the prognostic value of loss of staining for collagen type IV alpha 5 (COL4A5) and its relationship with the ultrastructural glomerular basement membrane alterations, we performed direct immunofluorescence using a mixture of fluorescein isothiocyanate-conjugated and Texas-red conjugated antibodies against COL4A5 and COL4A2, respectively, on renal biopsies of 25 males with AS (including 16 who were diagnosed in adulthood).

Results: All patients showed normal positive staining of glomerular basement membranes and tubular basement membranes for COL4A2. Of the 25 patients, 10 (40%) patients showed loss of staining for COL4A5 (including 89% of children and 13% of adults) and the remaining 15 (60%) had intact staining for COL4A5. Compared with patients with intact staining for COL4A5, those with loss of staining had more prominent ultrastructural glomerular basement membrane alterations and were younger at the time of biopsy. By Kaplan-Meier survival analysis and Cox regression analysis, loss of staining for COL4A5 predicted earlier progression to overt proteinuria and stage 2 chronic kidney disease or worse. By multivariate Cox regression analysis, loss of staining for COL4A5 was an independent predictor of the development of overt proteinuria and stage 2 chronic kidney disease or worse.

Discussion: Thus, the COL4A5 expression pattern has an important prognostic value and it correlates with the severity of ultrastructural glomerular basement membrane alterations in males with AS. Loss of COL4A5 staining is uncommon in patients with AS diagnosed in their adulthood.

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Alport syndrome (AS) (also known as Alport disease or hereditary nephritis) is a genetic disorder manifested by progressive hematuric nephropathy with or without sensorineural hearing loss and ocular lesions. The natural history of AS nephropathy is characterized by persistent microhematuria followed, in the later stages of disease, by proteinuria and then renal insufficiency. AS is due to mutations in one of the collagen type IV (COL4) genes that disrupt the synthesis of COL4 and/or the formation of COL4 protomers and networks. Multiple modes of inheritance are known. The X-linked type, which is due to mutations in the gene encoding the α5 chain of COL4 (COL4A5) present on chromosome X, is the most common. The remaining cases are autosomal recessive or autosomal dominant, and are due to mutations in the genes encoding α3 (COL4A3) or α4 (COL4A4) chains that are located on chromosome 2. *De novo* (sporadic) mutations

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may be responsible for cases that lack a family history of renal disease. The median renal survival of males with X-linked AS is 25 years, and 90% reach end-stage renal disease (ESRD) by the age of 40.[1] It has been shown that the renal prognosis is dependent, to a certain extent, on the underlying genetic defect, with truncating mutations leading to more severe disease and earlier progression to ESRD than missense mutations.[1,2] The development of proteinuria is a predictor of progression to ESRD in AS.[3]

There are no specific histologic alterations of AS on light microscopy. Early in the disease, glomeruli appear unremarkable or show a mild increase in mesangial matrix or mesangial cell number. Late in the disease, a pattern of focal segmental glomerulosclerosis may develop, associated with a variable degree of tubular atrophy and interstitial fibrosis (TA/IF). Interstitial foam cells are commonly identified, even before the development of heavy proteinuria. The diagnostic histologic finding in AS is seen on the ultrastructural examination of glomeruli. There are thickening, lamellation, and reticulation of the glomerular basement membranes (GBM), imparting a characteristic "basket-weave" appearance.[4] Scalloping of the outer aspect of the GBM and electron-dense microgranules, 20 to 60 nm in diameter, are frequently seen in the lucent areas between the GBM lamellations. These ultrastructural findings can be focal and segmental, and the unaffected segments may show GBM thinning. In some cases, especially in young boys and female heterozygotes with X-linked disease, the only ultrastructural finding may be diffuse thinning of the GBM.

Because of the large size of the COL4 genes and the numerous disease-causing mutations identified so far (1900 variants in *COL4A5* alone),[5] staining for COL4 chains has become a useful adjunct to establish the diagnosis and determine the mode of inheritance.[6–8] COL4 is composed of 6 chains, α1–α6, coiled around each other to form triple helical protomers, of which only 3 configurations have been discovered, α1.α1.α2.(IV), α3.α4.α5.(IV), and α5.α5.α6.(IV).[9] The triple helical protomers form hexamers, of which only 3 pairings exist: α1.α1.α2.(IV)–α1.α1.α2.(IV), α3.α4.α5.(IV)–α3.α4.α5.(IV), and α1.α1.α2.(IV)–α5.α5.α6.(IV).[9] The α1.α1.α2.(IV)–α1.α1.α2.(IV) network is expressed in GBM, mesangial matrix, Bowman's capsule (BC), and tubular basement membranes (TBM). The α3.α4.α5.(IV)–α3.α4.α5.(IV) network is expressed in mature GBM, BC, and distal TBM. The α1.α1.α2.(IV)–α5.α5.α6.(IV) network is expressed in BC and distal TBM. In X-linked AS, typically there is loss of COL4A5 staining of GBM, BC, and distal TBM, whereas in autosomal AS, there is loss of COL4A5 staining of GBM with intact staining of BC and distal TBM.[6,7,10] In contrast, patients with thin basement membrane nephropathy typically have intact GBM staining for COL4 subunits,[11] with the caveat that female carriers of AS often have thin GBM lesions and may show mosaic loss of staining.

Many previous studies have addressed the pathology, genetics, and treatment of AS; however, only a few, which included mostly children, have evaluated the prognostic value of COL4 chain staining[12,13] and the relationship between COL4 chain expression and ultrastructural GBM alterations.[8,14] In this study, we aim to address the following 3 questions: (1) Does loss of glomerular staining for COL4A5 have a prognostic value? (2) Does it correlate with the ultrastructural GBM alterations? and (3) Does staining for COL4A5 help in confirming the diagnosis of AS in cases that show only mild ultrastructural GBM alterations?

MATERIALS AND METHODS

We reviewed the renal pathology archives at the Mayo Clinic, from 2007 to 2015, and identified 28 males with AS who had fulfilled all of the following 3 criteria: (1) clinicopathologic diagnosis of AS including the presence of the defining ultrastructural finding of GBM basket weave change; (2) family history of hematuria (with or without renal failure) and/or hearing loss; and (3) adequate residual frozen tissue for immunofluorescence (IF) staining for COL4A5 and COL4A2. In light of findings of prior studies suggesting that patients with autosomal recessive AS have worse prognosis than those with X-linked AS,[15,16] we excluded 3 males who had an expression pattern consistent with autosomal AS from the outcome analysis. The remaining 25 patients were the subject of this study. Because most of our patients did not undergo genetic testing and because GBM lamellation similar to what is seen in AS has been described in severe podocytopathies including familial focal segmental glomerulosclerosis due to non-COL4 gene mutations,[17] we excluded 7 patients with typical electron microscopy (EM) findings of AS but without hearing loss or family history of hematuria (including 6 with preserved COL4A5 staining and 1 with loss of COL4A5 staining).

All renal biopsies were processed according to standard techniques for light microscopy, IF, and EM. For light microscopy, all cases were stained with hematoxylin and eosin, periodic acid-Schiff, Masson's trichrome, and Jones methenamine silver. IF was performed on 3- to 4-μm cryostat sections using polyclonal fluorescein isothiocyanate (FITC)-conjugated antibodies to IgG, IgM, IgA, C3, C1q, kappa, lambda, fibrinogen, and albumin (Dako Corporation, Carpinteria, CA). EM was performed on all cases. The median number of glomeruli studied ultrastructurally was 2 (range, 1–5).

We performed direct IF using a mixture of FITC-conjugated and Texas-red conjugated rat monoclonal antibodies against COL4A5 (clones H53 and B51) and COL4A2 (clone H25), respectively, as detailed in Table 1. This mixture of monoclonal antibodies is produced by Shigei Medical Research Institute (Okayama, Japan) and is commercially available. The H53 is specific to the IDVEF sequence at positions of 251–255 of COL4A5, B51 to the NC1 domain of COL4A5, and H25 to the EAIQP sequence at positions of 675–679 of COL4A2.[18,19] Patients showing loss of GBM, BC, and distal TBM positivity for COL4A5 were classified as group 1, whereas patients with intact GBM, BC, and distal TBM positivity for COL4A5 were classified as group 2 (Figure 1). The COL4A5 and COL4A2 IF slides were reviewed by 2 renal pathologists (MEF and SHN), who were blinded to EM findings. The results were recorded as positive versus negative staining of GBM, BC, and distal TBM for COL4A5 and COL4A2. There was 100% concordance rate between the 2 pathologists. When positive, GBM staining was bright (2+ or 3+, scale 0–3+), whereas BC and distal TBM staining ranged from 1 to 3+. Demographic information, presenting renal clinical and laboratory findings, and follow-up were obtained from patients' medical records and through conversations with the treating nephrologists. The age at detection of overt proteinuria (defined as 24-hour urine protein collection of >300 mg/day or protein-to-creatinine ratio > 300 mg/g creatinine) and the age at detection of stage 2 chronic kidney disease (CKD) or worse (i.e., estimated glomerular filtration rate < 90 ml/min per 1.73 m^2) were recorded. Clinical patient characteristics at the time of biopsy were also collected. Quantification of proteinuria was performed by 24-hour collection or by spot urine protein-to-creatinine ratio when 24-hour urine collection was not performed. Estimated glomerular filtration rate for children (≤18 years) was calculated by the revised Schwartz equation[20] and for adults by the Chronic Kidney Disease Epidemiology Collaboration equation[21] and expressed as ml/min per 1.73 m^2.

Table 1. Procedure for immunofluorescence staining for COL4A5 and COL4A2

1. Cut a 4-μm section from the frozen tissue onto a charged slide.
2. Place the slide in Dako Wash buffer (Dako, catalog # K8007) for ≥2 min.
3. Incubate with the prediluted mixture of FITC-conjugated and Texas-red conjugated rat monoclonal antibodies against COL4A5 (clones H53 and B51) and COL4A2 (clone H25) (Cosmo Bio USA, catalog # SGE-CFT45325) for 30 min at room temperature.
4. Place the slide in Dako wash buffer for ≥2 min.
5. Replace Dako wash buffer with distilled water to rinse for ≥2 min.
6. Mount in Vector-Hard-Set Mounting Medium (Vector, catalog # H-1400).
7. Examine the slide under a dark field ultraviolet (IF) microscope using a Texas Red/FITC combination filter and an FITC filter alone.

COL4A2, collagen type IV alpha 2; COL4A5, collagen type IV alpha 5; FITC, fluorescein isothiocyanate.

The severity of GBM ultrastructural alterations was graded as class 1: <25% of the total glomerular capillary surface area showing GBM basket weave change, lamellation and/or scalloping (with or without thinning of other loops), class 2: 25% to 75% of the total glomerular capillary surface area showing these findings, and class 3: >75% of the total glomerular capillary surface area showing these findings. The degree of podocyte foot process effacement was graded as 0 (none), 1% to 25% of the total glomerular capillary surface area showing effacement (mild), 26% to 50% (moderate), or >50% (severe). TA/IF were graded on a semiquantitative scale based on an estimate of the percentage of renal cortex affected and recorded as follows: 0 (none), 1% to 25% (mild), 26% to 50% (moderate), or >50% (severe). The histologic assessments including the EM findings were scored by 2 renal pathologists (SMS and SHN), who were blinded to the diagnosis and COL4A5 IF staining results. For scoring the EM class, there was concordance in 21 cases and discordance in 4 cases (class 2 vs. 1 in 3 cases and class 3 vs. 2 in 1 case). A consensus score was used in these 4 cases. For assessing the percentage of total peripheral capillary surface area with podocyte foot process effacement, there was concordance in 23 cases and discordance in 2 cases. A consensus score was used in these 2 cases.

Statistical analyses were performed using SPSS for Windows, version 17.0 (SPSS, Chicago, IL) and StatXact for Windows, version 11.0 (Cytel Software Corporation, Cambridge, MA). Continuous variables are reported as the mean ± SEM. Analyses were performed by nonparametric exact statistical methods using the Wilcoxon rank-sum test, the Kruskal-Wallis test, the Jonckheere-Terpstra test, and the Fisher exact test, as appropriate for variable type. Survival estimates (endpoints, overt proteinuria, stage 2 CKD or worse) were computed by the method of Kaplan and Meier. Multivariate survival analyses were performed using the Cox proportional hazards model (Cox regression). Statistical significance was assumed at $P < 0.05$. P values between 0.05 and 0.10 were considered to be of borderline significance. The study was approved by the Mayo Clinic Institutional Review Board.

RESULTS

Expression Patterns of COL4A5 Staining

All of the 25 cases studied showed normal diffuse positive staining of GBM, BC, and TBM for COL4A2. In 10 cases (40%) (group 1), there was loss of distal TBM staining for COL4A5. Of these 10 cases, 2 showed segmental loss of GBM staining for COL4A5 (mosaic pattern), 7 showed global loss of GBM staining for COL4A5, and 1 did not have glomeruli left in the frozen

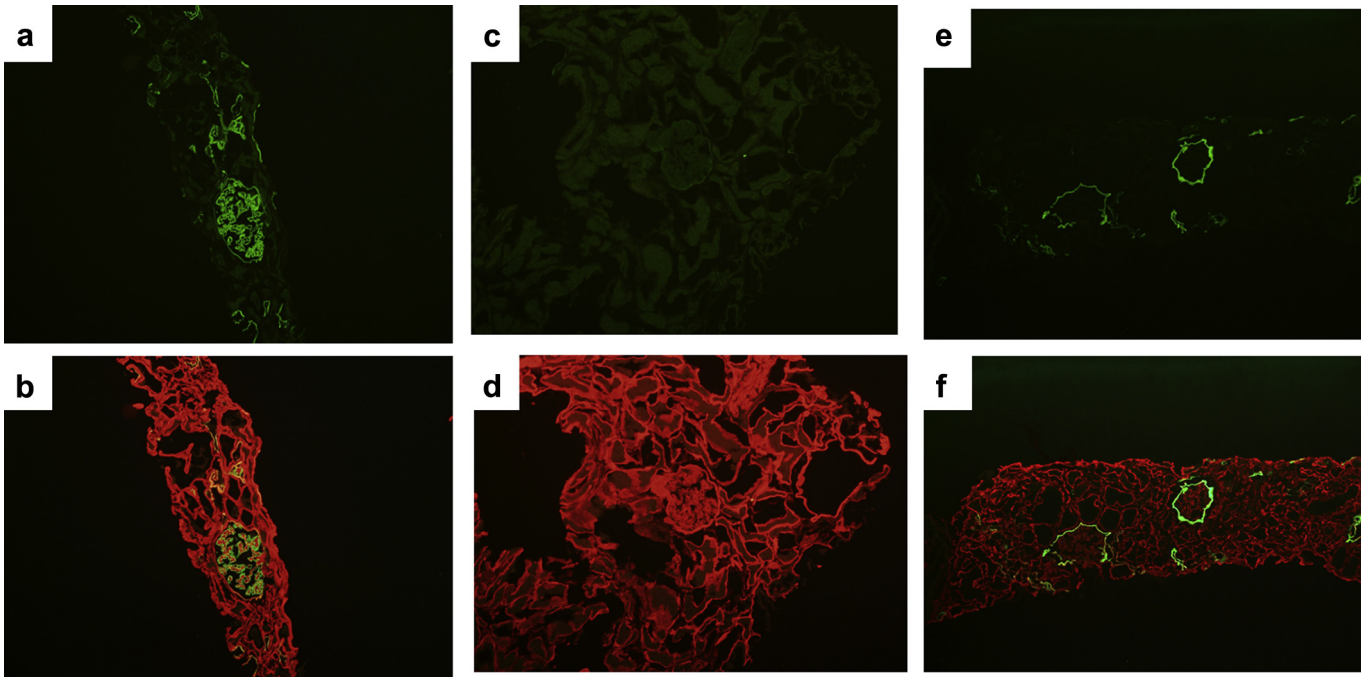

Figure 1. COL4A5 expression patterns. (a,b) Images (same microscopic field) from a patient in group 2 showing intact GBM, BC, and distal TBM staining for COL4A5. (c,d) Images (same microscopic field) from a patient in group 1 showing complete loss of staining of GBM, BC, and distal TBM for COL4A5, consistent with X-linked Alport syndrome. (e,f) Images (same microscopic field) showing complete loss of staining of GBM for COL4A5 with intact staining of BC and distal TBM, consistent with autosomal Alport syndrome. Images (a), (c), and (e) were visualized under the FITC immunofluorescence filter, whereas images (b), (d), and (f) were visualized under the Texas Red/FITC combination immunofluorescence filter. Immunofluorescence was performed using a mixture of FITC-conjugated and Texas-red conjugated rat monoclonal antibodies against COL4A5 and COL4A2, respectively. All cases showed the normal diffuse staining of GBM, BC, and TBM for COL4A2 (red color in b, d, and f). (Original magnification ×200 for a–d and ×100 for e and f.) BC, Bowman's capsule; COL4A2, collagen type IV alpha 2; COL4A5, collagen type IV alpha 5; FITC, fluorescein isothiocyanate; GBM, glomerular basement membrane; TBM, tubular basement membrane.

tissue. The remaining 15 (60%) cases (group 2) showed intact GBM, distal TBM, and BC staining for COL4A5 (Figure 1).

Pathologic Findings

The mean number of glomeruli sampled for light microscopy and the percentage of globally sclerotic glomeruli for the entire cohort were 16 (range, 1–69) and 14% (range, 0%–50%), respectively. Segmental glomerulosclerosis was present in 36% of cases and interstitial foam cells in 36% of cases. The degree of TA/IF was none in 40%, mild in 44%, moderate in 8%, and marked in 8%. The EM class was 1 in 28% of cases, 2 in 20%, and 3 in 52%. Table 2 shows the statistically significant pathologic differences between group 1 and group 2. Compared with group 2, group 1 had a lower percentage of global glomerulosclerosis and lower degree of arteriosclerosis, likely reflecting their younger age at biopsy (see later). Group 1 had more prominent ultrastructural GBM abnormalities (mean EM class 2.8 vs. 1.9, $P = 0.01$) (Figure 2a–d). None of the patients in group 1 had class 1 ultrastructural findings (vs. 47% of patients in group 2, $P = 0.02$). There were no statistical differences between the 2 groups with regard to the presence of segmental sclerosis lesions, presence of interstitial foam cells, degree of TA/IF, or degree of podocyte foot process effacement (data not shown). One patient in group 2 had concurrent mild IgA nephropathy. Another patient in group 2 who had ESRD at biopsy (performed at the age of 34 years) showed concurrent chronic interstitial nephritis with marked TA/IF.

Clinical Findings and Outcome

The cohort consisted of 25 males; 19 (76%) were white, 3 (12%) Hispanic, 2 (8%) Asian, and 1 (4%) was African American. The study included 3 brothers (all

Table 2. Statistically significant pathologic differences between patients with (group 1) or without (group 2) loss of staining for COL4A5

	Group 1	Group 2	*P* value
No. of cases	10	15	
% global glomerulosclerosis	5.6 ± 8.1	19.5 ± 12.9	0.003
≥ mild arteriosclerosis	2 (20%)	11 (73%)	0.01
EM class			0.01
1	0	7 (47%)	
2	2 (20%)	3 (20%)	
3	8 (80%)	5 (33%)	

Data are expressed in mean ± SD or number (percentage) of cases.
COL4A5, collagen type IV alpha 5; EM, electron microscopy.

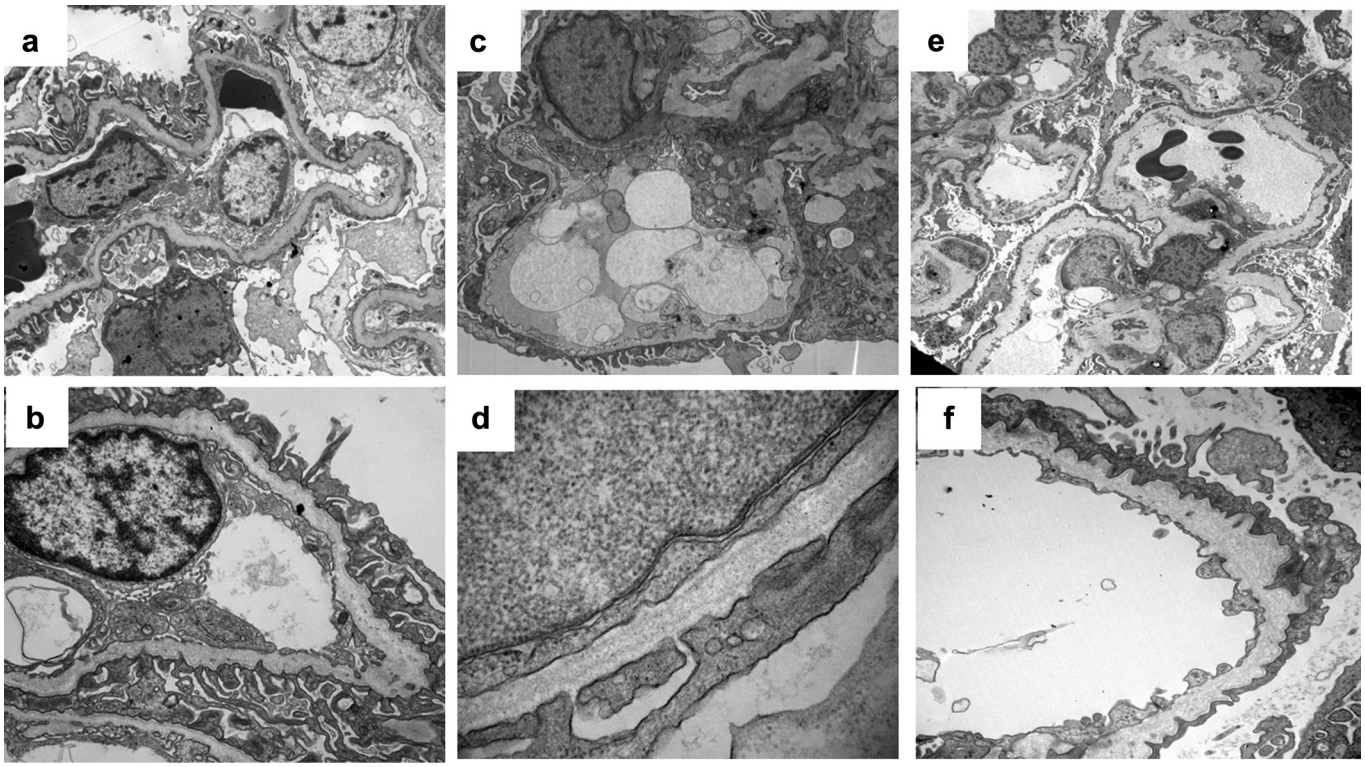

Figure 2. Electron microscopic findings. (a,b) Images from a 7-year-old boy with a family history of hematuria in his mother and grandmother. Electron microscopy shows global thickening, basket weave splitting, and scalloping of the GBM (×4800 for a and ×13,000 for b). In this case, there was complete loss of GBM, BC, and distal TBM staining for COL4A5. (c,d) Images from a 14-year-old boy with hearing loss and a family history of Alport syndrome in his sister and grandmother. Electron microscopy showed mostly diffuse thinning of the GBM (c, ×5800), but rare GBM segments showed lamellation (d, ×59,000). In this case, there was intact GBM, BC, and distal TBM staining for COL4A5. (e,f) Images from a 25-year-old man with a family history significant for proteinuria and hematuria in his mother and end-stage renal disease in his grandmother. Electron microscopy showed global thickening, basket weave splitting, and scalloping of the GBM (×2900 for e and ×11,000 for f). In this case, there was intact GBM, BC, and distal TBM staining for COL4A5. BC, Bowman's capsule; COL4A5, collagen type IV alpha 5; GBM, glomerular basement membrane; TBM, tubular basement membrane.

in group 1), and the remaining 22 patients were unrelated. All patients had a family history of hematuria (with or without renal insufficiency) (present in 80%) and/or hearing loss (present in 60%). At the time of renal biopsy, 9 (36%) patients were children and 16 (64%) were adults, with a mean age of 29 ± 17 years (range, 7–67 years), a mean serum creatinine of 1.6 ± 2.1 mg/dl, a mean estimated glomerular filtration rate of 80 ± 36.5 ml/min per 1.73 m^2, a mean 24-hour urine protein of 2.0 ±1.9 g, and a mean serum albumin of 3.8 ± 0.5 g/dl. At biopsy, 96% of the patients had microscopic hematuria, 36% hypertension, 83% overt proteinuria, and 56% stage 2 CKD or worse; none had full nephrotic syndrome. Table 3 shows the statistically significant clinical differences between patients in group 1 and group 2. Patients in group 1 were younger at biopsy and less likely to be white, and had a higher estimated glomerular filtration rate and lower serum creatinine at biopsy likely reflecting younger age at biopsy. Degree of proteinuria at biopsy, presence of hypertension, level of serum albumin at biopsy, presence of family history, and presence of hearing loss were not statistically different between the 2 groups. Macroscopic hematuria tended to be more common in group 1 ($P = 0.08$). Post kidney biopsy follow-up was available in 72% of patients (mean 35 months [31 months for group 1 and 38 months for group 2], range 7–102 months). During the follow-up period, 2 patients (in group 2) developed ESRD at ages 34 and 47, respectively.

Table 3. Statistically significant clinical differences between patients with (group 1) or without (group 2) loss of staining for COL4A5

	Group 1	Group 2	*P* value
No. of patients	10	15	
Age at Bx	14.4 ± 7.0	38.7 ± 15.0	<0.001
Age at detection of overt proteinuria	12.4 ± 6.7	30.8 ± 16.1	<0.001
Age at detection of stage 2 CKD or worse	15.6 ± 6.3	37.3 ± 13.2	<0.001
White race	4 (40%)	15 (100%)	0.001
Serum creatinine at Bx in mg/dl	0.9 ± 0.7	2.0 ± 2.5	0.007
eGFR at Bx	99.4 ± 34.5	67.1 ± 32.7	0.03
Macroscopic hematuria	5 (50%)	2 (13%)	0.075

Data are expressed in mean ± SD or number (percentage) of cases.
Bx, renal biopsy; CKD, chronic kidney disease; COL4A5, collagen type IV alpha 5; eGFR, estimated glomerular filtration rate.

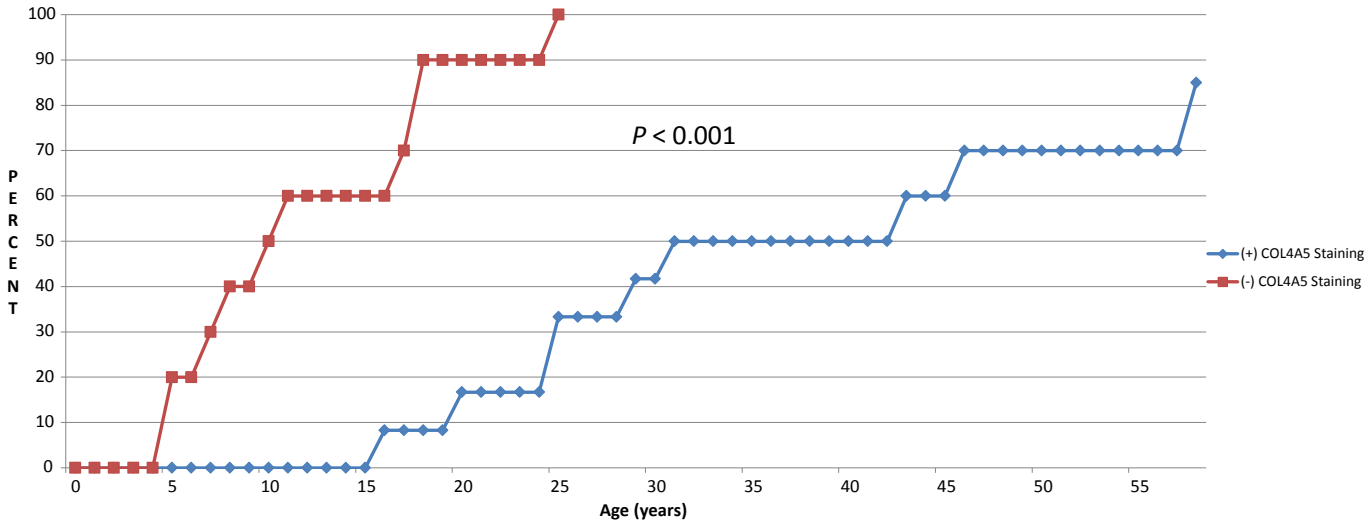

Figure 3. Progression to overt proteinuria. COL4A5, collagen type IV alpha 5.

Importantly, compared with group 2 patients, group 1 patients developed overt proteinuria and stage 2 CKD or worse at a younger age ($P < 0.001$ for both) (Table 3). By Kaplan-Meier survival analysis and Cox regression analysis, loss of staining for COL4A5 predicted earlier progression to overt proteinuria ($P < 0.001$, median age 10 years [95% confidence interval (CI), 5.1–14.6] vs. 31 years [9.4–52.6]) and to stage 2 CKD or worse ($P < 0.001$, median age 18 years [95% CI, 14.7–21.3] vs. 40 years [26.4–54.0]) (Figures 3 and 4). There were not enough endpoints to calculate for progression to ESRD. The presence of EM class 2 or 3 findings (vs. class 1) also predicted earlier progression to overt proteinuria ($P < 0.003$, median age 18 years [95% CI, 9.3–26.7] vs. 58 years [26.9–89.1]) and to stage 2 CKD or worse ($P < 0.007$, median age 29 years [95% CI, 14–44] vs. 47 years [CI, 25.4–68.6]) (Figures 5 and 6).

In multivariate Cox regression analysis for all variables, independent predictors of progression to overt proteinuria were loss of staining for COL4A5 and % global glomerulosclerosis; the only independent predictor for progression to stage 2 CKD or worse was loss of staining for COL4A5 (Table 4).

DISCUSSION

This is the first study on AS to include mostly adults at diagnosis. We have shown that loss of staining for COL4A5 has prognostic value as it correlates with earlier onset of proteinuria and stage 2 CKD, likely reflecting the presence of large genetic defects and hence major COL4 network alterations in these patients. Two previous studies addressing mostly children with X-linked AS have found correlation between loss of staining and earlier onset of proteinuria and

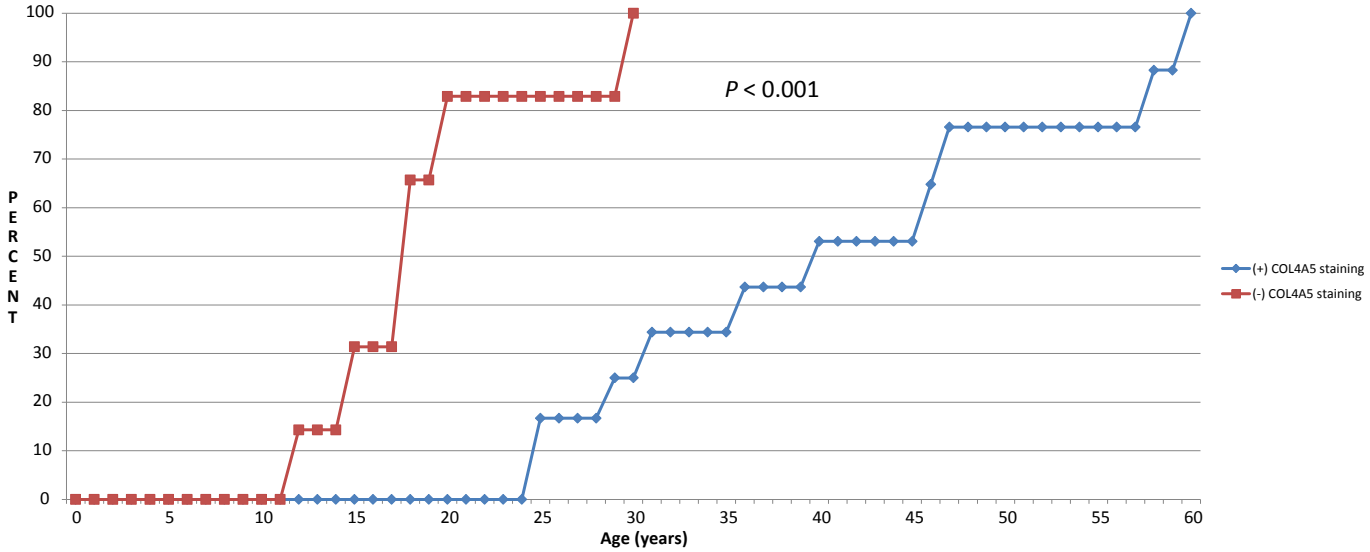

Figure 4. Progression to CKD2 or worse. CKD2, stage 2 chronic kidney disease; COL4A5, collagen type IV alpha 5.

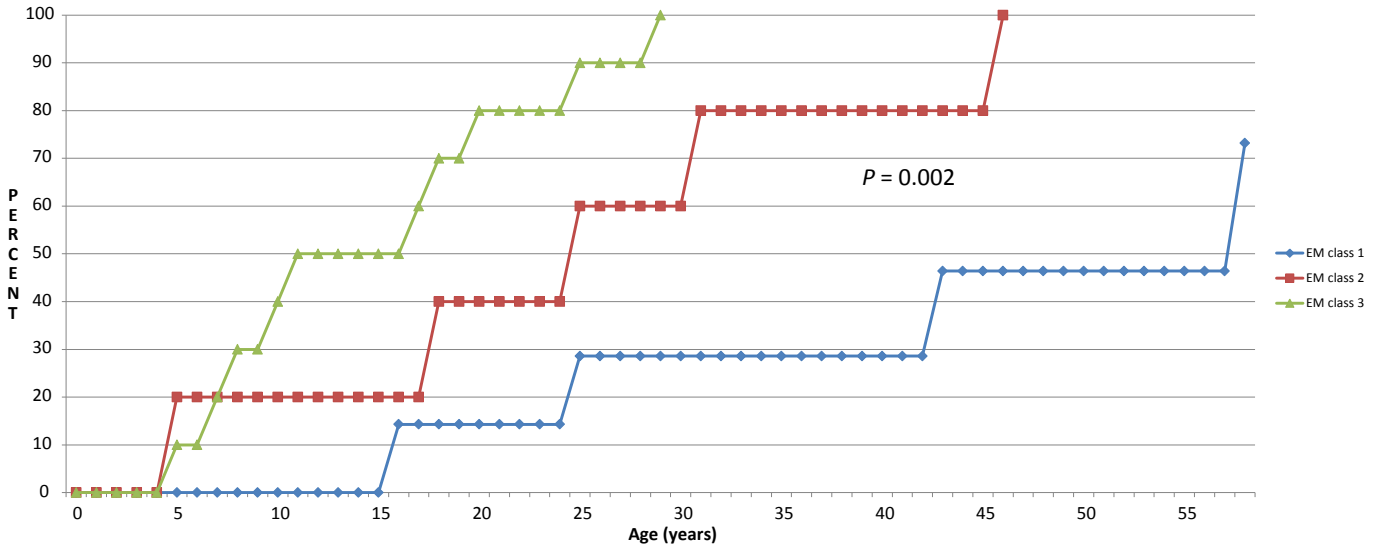

Figure 5. Progression of overt proteinuria according to EM class. EM, electron microscopy.

ESRD.[12,13] It has been shown in males with X-linked AS that truncating mutations (nonsense, insertion, deletion, splice site) in the *COL4A5* gene typically lead to loss of GBM staining for COL4A5, whereas missense mutations can be associated with loss or intact staining.[12,22] Based on the above data, staining for COL4A5 is recommended in the practice of renal pathology in patients with a histologic diagnosis of AS. The staining technique that we use in our laboratory (detailed in Table 1) and that is used in several other diagnostic renal pathology laboratories (such as Arkana Laboratories and the Immunopathology Laboratory of University of Minnesota) and in several prior studies[12,18,22] is simple, quick, and inexpensive as it is a direct IF method involving incubating a single frozen tissue section with a commercially available prediluted mixture of FITC-conjugated COL4A5 and Texas-red conjugated COL4A2 antibodies. It does however require the addition of a Texas Red/FITC combination filter to the routine IF microscope. The combination filter is particularly useful to diagnose cases with mosaic loss of COL4A5 staining, seen sometimes in females; in these cases, GBM segments negative for the FITC-conjugated COL4A5 antibody appear red as they retain their normal COL4A2 positivity.

The frequencies of X-linked, autosomal recessive, and autosomal dominant AS were previously estimated to be 80% to 85%, 15%, and 1% to 5%, respectively.[23,24] However, recent studies using next-generation sequencing for mutation screening of *COL4A5*, *COL4A4*, and *COL4A3* have identified a higher frequency of autosomal AS, particularly the autosomal dominant type that accounted for 19% to 30% of cases.[25,26] In our experience with 17 patients

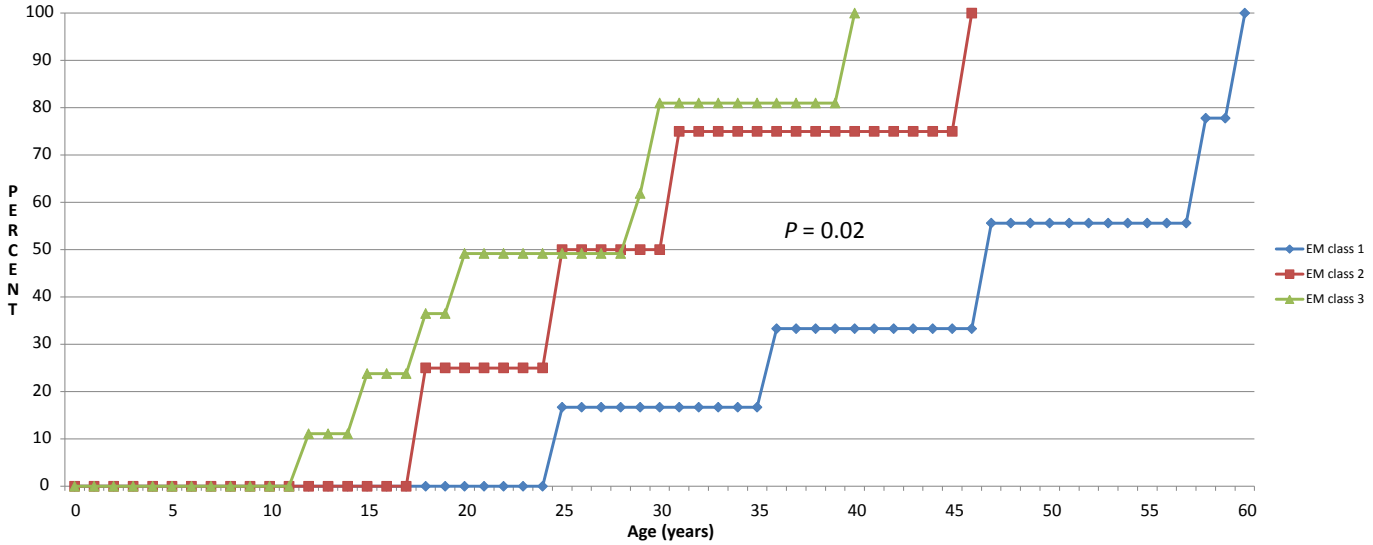

Figure 6. Progression to CKD2 or worse according to EM class. CKD2, stage 2 chronic kidney disease; EM, electron microscopy.

Table 4. Multivariate Cox regression analysis

	HR	95% CI	*P* value
Progression to overt proteinuria			
(–) versus (+) COL4A5 staining	10.17	2.43–42.49	0.001
% global glomerulosclerosis	0.93	0.87–0.99	0.017
Progression to stage 2 CKD or worse			
(–) versus (+) COL4A5 staining	11.35	2.66–48.53	0.001

CI, confidence interval; CKD, chronic kidney disease; COL4A5, collagen type IV alpha 5; HR, hazard ratio.

with AS with loss of COL4A5 staining (including males and females), 11 (65%) showed an expression pattern consistent with X-linked disease whereas 6 (35%) showed an expression pattern consistent with autosomal disease (example of the latter is shown in Figure 1e and f), which is another reason for performing COL4A5 staining in patients suspected to have AS, particularly those who cannot afford or are unwilling to undergo genetic testing. Even in patients with confirmed mutations in *COL4A5*, *COL4A4*, or *COL4A3,* COL4A5 expression evaluation is still warranted as some mutations may not be pathogenic and, as was shown recently, heterozygous mutations in *COL4A4* or *COL4A3* are present in 10% of patients with familial focal segmental glomerulosclerosis (with EM findings inconsistent with classic AS).[27]

Previous studies on AS that included 90% to 100% children have found loss of staining for COL4A5 in over two-thirds of patients.[12–14,24] In contrast, in our renal biopsy-based series in which 64% of patients were adults at biopsy, only 40% of patients showed loss of staining for COL4A5, including 89% (8 of 9) of children and 13% (2 of 16) of adults. The high percentage of adults in our cohort reflects our general renal biopsy practice in which the vast majority of biopsies are from adults, with pediatric biopsies accounting for only 2% of our native renal biopsies. Similar to our experience, only 38% (6 of 16) of the patients tested by Markowitz *et al.*[7] who were mostly adults at biopsy had loss of GBM staining for COL4A5. As patients with loss of staining for COL4A5 typically develop proteinuria and stage 2 CKD at an early age, they tend to undergo diagnostic renal biopsy during childhood. Hence, only a small minority of patients with AS diagnosed in adulthood are expected to have loss of staining for COL4A5, a point that has not been addressed in the literature.

In this study, we found a correlation between the loss of staining for COL4A5 and the severity of ultrastructural GBM alterations. None of the cases that showed mild ultrastructural alterations (i.e., focal GBM basket weave change, with or without thinning of other loops) showed loss of staining for COL4A5. As illustrated in Figure 2e and f, even cases with striking global GBM basket weave change may show intact staining for COL4A5, and therefore EM is more sensitive than immunostaining for COL4A5 to diagnose AS. Because we studied only males who were mostly diagnosed with AS in adulthood, caution is warranted in extrapolating our results to children and females.

This work has some noteworthy limitations. (1) It is a retrospective study with a small sample size. (2) Only 2 (8%) patients developed ESRD. This is likely in part due to the lack of long-term follow-up. Because most of our patients were diagnosed in adulthood and had intact staining for COL4A5, the low incidence of ESRD may also be a reflection of milder genetic defects in these patients (i.e., selection bias). (3) Genotype-phenotype correlations were not possible as the majority of patients included in this study did not undergo genetic testing. Several previous studies have addressed the relationship between COL4A5 mutations and the pattern of COL4A5 staining in X-linked AS.[3,12,13,22] With the recent improved mutational screening through next-generation sequencing, future studies evaluating the relationship between *COL4A4* and *COL4A3* mutations and the pattern of COL4A5 staining in autosomal AS will likely become possible.

In this study, we show that loss of staining for COL4A5 correlates with more severe GBM ultrastructural alterations, a younger age at diagnosis, and earlier progression to overt proteinuria and stage CKD2 or worse, likely reflecting larger genetic defects in these patients; hence, staining for COL4A5 provides important prognostic information in patients with AS.

DISCLOSURE

All the authors declared no competing interests.

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