Kidney360

A COL4A4-G394S Variant and Impaired Collagen IV Trimerization in a Patient with Mild Alport Syndrome

Jennefer Kohler [1], *Kohei Omachi* [2], *Vivek Charu* [3], *Jeffrey H. Miner* [2], *and Vivek Bhalla* [4]

Key Points

- Missense variants in *COL4A* genes can cause mild forms of Alport syndrome.
- Combining pathology and genetics with basic science can successfully determine the pathogenicity of variants of uncertain significance.
- Low-throughput, mechanistic approaches, applied broadly, may provide a critical next step for precision and personalized medicine.

Abstract

Background Missense variants in *COL4A* genes are often found in patients with an Alport syndrome–like presentation, but their pathogenicity is not always clear. We encountered a woman with microscopic hematuria and proteinuria at 33 years of age with a diagnosis of thin basement membrane disease who was approaching end stage kidney disease at 59 years of age. We hypothesized that this patient's kidney disease was within the spectrum of Alport syndrome.

Methods We used histologic, genetic, and biochemical approaches to investigate the mechanisms of kidney disease. By immunofluorescence, we investigated collagen IV chain composition of the glomerular basement membrane (GBM). We employed targeted sequencing to search for pathogenic variants in *COL4A* and other relevant genes. We utilized N- and C-terminal split NanoLuciferase assays to determine the effect of a novel *COL4A4* variant of uncertain significance (VUS) on collagen IV heterotrimer formation *in vitro*. We transfected COL4A4 expression constructs with split NanoLuciferase fragment-fused COL4A3 and COL4A5 constructs into human embryonic kidney 293T cells. To assay for α3α4α5(IV) heterotrimer formation and secretion, we measured luminescence in cell lysates and culture supernatants from transfected cells.

Results Immunostaining suggested that the collagen α3α4α5(IV) network was present throughout the patient's GBMs. DNA sequencing revealed a novel homozygous VUS: *COL4A4* c.1180G>A (p. Gly394Ser). In the C-terminal split luciferase-based α3α4α5(IV) heterotrimer formation assays, luminescence levels for G394S were comparable to WT, but in the N-terminal tag assays, the extracellular luminescence levels for G394S were decreased by approximately 50% compared with WT.

Conclusions Our cell-based assay provides a platform to test *COL4* VUS and shows that G394S impairs assembly of the α3α4α5(IV) N-terminus and subsequent trimer secretion. These data suggest that the COL4A4-G394S variant is pathogenic and causes an atypical mild form of autosomal recessive Alport syndrome.

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Introduction

Alport syndrome is an inherited kidney disorder characterized by hematuria, proteinuria, and progressive kidney failure, and hearing loss and ocular anomalies (1, 2). Disease features, age at onset, and severity of progression vary widely. In recent years, Alport syndrome has been identified genetically in individuals clinically diagnosed with focal segmental glomerulosclerosis and IgA nephropathy (3, 4). Alport syndrome is among the most common inherited kidney disorders, with an estimated prevalence of 1:5000 in the general population, although this may be an underestimate (5).

Alport syndrome results from collagen IV defects that lead to thinning, lamellation, and thickening of the glomerular basement membrane (GBM) and to dysfunction of the collagen IV networks in the cochlea and eye. The syndrome is caused by pathogenic variants in the *COL4A3*, *COL4A4*, and *COL4A5* genes that encode the α3, α4, and α5 chains, respectively, comprising the α3α4α5(IV) heterotrimer (6, 7). Pathogenic

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variants disrupt the normal structure of the α3α4α5(IV) network through improper chain or heterotrimer folding (as with missense variants) or degradation of heterotrimer components (as with protein-truncating variants and deletions) (6). Depending on genetic etiology, Alport syndrome may be inherited in X-linked (*COL4A5*) or autosomal recessive or autosomal dominant (*COL4A3*, *COL4A4*) modes (8). In rare cases, digenic inheritance has also been reported (9).

Disease severity and progression are influenced by an individual's genotype (10). Individuals with autosomal recessive Alport syndrome and XY individuals with X-linked disease typically have a severe clinical course, resulting in kidney failure in adolescence or by early to mid-adulthood. By contrast, those with autosomal dominant disease and XX individuals with X-linked disease typically have a milder disease course, with lower risk for progression to kidney failure, and they may even present with isolated hematuria but without proteinuria, kidney dysfunction, hearing loss, or ocular findings. Individuals with heterozygous *COL4A3* or *COL4A4* mutations with hematuria are often classified as having thin basement membrane nephropathy (TBMN) rather than Alport syndrome, although it is recommended that these individuals be considered part of the Alport spectrum because a significant percentage will progress to proteinuria, focal segmental glomerulosclerosis, and ESKD later in life (11, 12). Despite the known influence of *COL4A* genotypes on disease severity, these generalizations do not account for individual-specific modifying factors or for the possibility of hypomorphic variants, which are associated with a more attenuated clinical course (4).

Genetic testing for Alport syndrome identifies a causative variant in approximately 80% of cases for which Alport syndrome is suspected clinically (13). However, variants of uncertain significance (VUS) remain a prevalent finding from *COL4A3*, *COL4A4*, and *COL4A5* sequencing. Approximately half of the variants identified in these genes by diagnostic laboratories are novel, and particularly in the case of missense variants, many lack the evidence needed to determine pathogenicity (4). *In silico* prediction models are prone to overestimate consequences of VUS in Alport genes (14) and are therefore limited in their ability to clarify variant pathogenicity.

Here, we present histologic, genetic, and biochemical investigation of the mechanisms of kidney disease in a case with CKD and a homozygous VUS in the *COL4A4* gene.

Materials and Methods

This study was approved by the Institutional Review Board at the Stanford University School of Medicine.

Biopsy Processing

Standard processing of kidney biopsies included light microscopy, immunofluorescence, and electron microscopy. For light microscopy, biopsy specimens were stained with hematoxylin and eosin, periodic acid–Schiff, and Jones methenamine silver. For immunofluorescence, cryostat sections were stained with polyclonal FITC-conjugated antibodies to IgG, IgM, IgA, C3, C1q, κ and λ light chains, fibrinogen, and albumin as per routine clinical testing. Electron microscopy was performed as per routine clinical practice. Additional immunofluorescence was performed for type IV collagen α2 (Texas red conjugated; Cosmo Bio, Carlsbad, CA), α3 (FITC conjugated; Weis Lab, Winston-Salem, NC), and α5 (FITC conjugated; Cosmo Bio) chains as part of clinical care.

In addition, fresh-frozen sections were immunostained with rat anti-human COL4A4 (clone H43; Chondrex, Inc., Woodinville, WA) plus rabbit anti-laminin-111 (L9393; Sigma–Aldrich, St. Louis, MO) followed by appropriate secondary antibodies. For COL4A4 staining, sections were treated with urea-glycine as previously described (15).

Sequencing

Using a saliva sample, we ordered analysis of a multigene panel, including the *COL4A3*, *COL4A4*, and *COL4A5* genes from a CLIA-certified genetic testing laboratory. Testing involved next-generation sequencing of 17 genes associated with progressive kidney disease (Supplemental Table). Analysis was focused on the coding exons and 10 bases of flanking intronic sequences for each gene. The commercial laboratory evaluated deletions and duplications on the basis of depth of coverage. Given limitations in next-generation sequencing-based deletion/duplication calling, we also ordered an exon-level oligo array comparative genomic hybridization encompassing the *COL4A3* and *COL4A4* genes.

Cell Culture

HEK293T cells (ATCC CRL-3216) were maintained at 37°C, 5% CO_2 in DMEM supplemented with 10% heat-inactivated FBS and penicillin-streptomycin on tissue culture plates coated with rat tail collagen I (#A1048301; Gibco, Grand Island, NY).

Plasmids

The human COL4A3-SmBiT (C-terminal tag), COL4A5-LgBiT (C-terminal tag), COL4A3-SmBiT (N-terminal tag), and COL4A5-LgBiT (N-terminal tag) coding sequences were amplified from pFC36K-SmBiT-COL4A3, pFC33K-LgBiT-COL4A5, pFN35K-SmBiT-COL4A3 and pFN33K-LgBiT-COL4A5 plasmids (16) and inserted into the pLV-EF1a-Hyg or pLV-EF1a-BSD lentiviral vector. LV-EF1a-Zeo was created by replacing the BSD segment of pLV-EF1a-BSD with the Zeocin resistance gene, and the Luc2 coding sequence was inserted into pLV-EF1a-Zeo.

Site-Directed Mutagenesis

pLV-Puro COL4A4 was generated from pLV-Puro COL4A4–3xFLAG (16) by site-directed mutagenesis with mutagenesis primers (sense 5′-ggtctgcgtgaagtatagctAggacta taaggaccacgacgga-3′ and antisense 5′- tccgtcgtggtccttatagtcc Tagctatacttcacgcagacc-3′). Subsequently, the three patient-derived variants were introduced into pLV-Puro-COL4A4 by site-directed mutagenesis. Briefly, pLV-COL4A4 was PCR amplified with high fidelity PrimeSTAR Max DNA polymerase (#R045A; Takara Bio, Mountain View, CA) and the following mutagenesis primers: G394S, sense 5′-ccccc aggtctcttgAgcagaccaggggaagcctg-3′, antisense 5′ caggcttcccc tggtctgcTcaagagacctggggg-3′; G545A, sense 5′-gggctaccagg aaagcatgCtgcctctggaccacct-3′, antisense 5′-aggtggtccagaggc aGcatgctttcctggtagccc-3′; and G960R, sense 5′-caaaggagcc ataggacctcccCgagatgaaggagaaatggctatc-3′, antisense 5′-gata

gccatttctccttcatctcGgggaggtcctatggctcctttg-3′. The PCR products were purified with a QIAquick PCR purification kit (#28106; Qiagen, Hilden, Germany), and the remaining plasmid template DNA was digested with *Dpn*I (#R0176S; New England Biolabs, Ipswich, MA). The amplicons were transformed into *Escherichia coli* to obtain variant plasmids. Successful mutagenesis was confirmed by Sanger sequencing (GENEWIZ/Azenta Life Sciences, South Plainfield, NJ). The primers used for sequencing were primer 1 (for G394S and G545A), 5′-ttctccagcatgtccatccc-3′, and primer 2 (for G960R), 5′-ggcattcctggattcctaggtc-3′.

Lentivirus Production, Transduction, and Generation of Stable Cell Lines

To produce lentivirus, 293T packaging cells were seeded at $5.5–6.0 \times 10^5$ cells per well in DMEM on six-well plates. Twenty hours after seeding, culture media were changed to fresh DMEM with 10% FBS and incubated with 1 μg of psPAX2 (#12260; Addgene, Watertown, MA), 100 ng of pMD2.G (#12259; Addgene), 1 μg of lentivirus transfer vector, and Lipofectamine 3000 transfection reagent (Invitrogen, Carlsbad, CA). Twenty-four hours after transfection, culture media was changed to DMEM supplemented with 30% FBS. The transfected cells were cultured for 24 hours, and the supernatant containing lentivirus particle was collected and filtered through a 0.45-μm syringe filter (polyvinylidene fluoride or polyether sulfone membrane). For lentivirus transduction, HEK293T cells were seeded in lentivirus containing media supplemented with 8 μg/ml polybrene (Sigma–Aldrich) on six-well plates and cultured for 24 hours. Then, cells were cultured in DMEM with 10% FBS. Lentivirus-transduced cells were selected by treating with the appropriate antibiotics for 2 weeks (hygromycin: 200–400 μg/ml; blasticidin: 10 μg/ml; puromycin: 10 μg/ml; zeocin: 300 μg/ml).

Dual Luciferase Assays

COL4A4 expression plasmids (wild type [WT], G394S, G545A, and G960R) were transiently transfected using FuGENE HD into Luc/COL4A3-SmBiT/COL4A5-LgBiT triple stable HEK293T cells. Twenty-four hours after transfection, cells were replated on Nunc 96-well white culture plates (#136101; Thermo Fisher Scientific, Waltham, MA). Twenty-four hours after replating, culture media was changed to phenol red-free DMEM (#11054020; Thermo Fisher Scientific) supplemented with 10% FBS, GlutaMax, penicillin-streptomycin, and 50 μM ascorbic acid and incubated for an additional 24 hours. Supernatants were transferred to new wells, and cells were washed with PBS and then provided with fresh PBS. The NanoGlo dual-luciferase reporter assay system (N1620; Promega, Madison, WI) was used to measure the intracellular Firefly luciferase activity as an internal control, followed by intracellular and extracellular NanoLuc luciferase assays.

Statistical Analyses

NanoGlo dual-luciferase reporter assays were performed in quadruplicate, starting from four separate cell cultures. All data are presented as the mean±SEM. The significance of differences was assessed using ANOVA with Dunnett's tests. Differences with $P < 0.05$ were considered statistically significant.

Results

Case Report

The patient is a 59-year-old South Asian woman followed at the Stanford Healthcare Ambulatory Nephrology Practice for a history of progressive kidney disease. She first presented at the age of 33 years with hematuria and proteinuria. By the age of 59 years, she had progressed to stage IV CKD. Family history was not significant for CKD, kidney failure, known hematuria, or proteinuria. One paternal uncle was on dialysis for 10 days in his ninth decade of life, but treatment was discontinued after resolution of AKI.

The patient has had two kidney biopsies: one at the age of 36 and the other at the age of 49 years (Figure 1). By histology, the first biopsy demonstrated primarily unremarkable glomeruli, with 18% global glomerulosclerosis (four globally sclerosed glomeruli of 22 sampled), and minimal interstitial fibrosis and tubular atrophy (5%). Ultrastructural examination demonstrated marked, but segmental, thinning of the GBM, with an overall thickness of 160–170 nm, suggesting the diagnosis of TBMN. In some areas, the GBMs were of normal thickness (approximately 300 nm). Classic ultrastructural features of Alport syndrome, including multilamellation and "basket-weaving" of the GBM and "subepithelial scalloping," were not appreciated. These findings raised the possibility of TBMN or a related disease within the spectrum of Alport syndrome.

Due to the onset of diabetes and progressive kidney disease, the patient underwent a second kidney biopsy approximately 13 years later. This biopsy demonstrated progressive global glomerulosclerosis with focal segmental glomerulosclerosis and increased interstitial fibrosis and tubular atrophy (20%–25%). Seven of 23 (30%) glomeruli sampled demonstrated global glomerulosclerosis, and three glomeruli demonstrated segmental sclerosis. Ultrastructural examination of the glomeruli again demonstrated marked, but segmental, thinning of the GBM, measuring as little as 150 nm in some areas. Segmental hyalinosis/sclerosis lesions were seen. As in the prior biopsy, conventional ultrastructural features of Alport syndrome were not appreciated. Neither biopsy demonstrated significant mesangial matrix expansion or GBM thickening suggestive of diabetic nephropathy. In both biopsies, only patchy foot process effacement was seen, lending no support for a primary podocytopathy.

Because TBMN is within the spectrum of Alport syndrome, we immunostained frozen sections with antibodies to the α1/2, α4, and α5 chains of type IV collagen. An antibody to laminin-111 was used as a counterstain. The pattern within the glomerular tuft was similar to controls, with α4 and α5 in the GBM and distal tubular basement membranes (and α5 in Bowman's capsule) and α1/2 widely expressed but not prominent in the GBM (Figure 2). The detection of α4 and α5 chains in the GBM illustrates the presence of intact collagen $\alpha3\alpha4\alpha5$(IV) heterotrimers. However, this staining does not address their quantity or quality and does not rule out the spectrum of Alport syndrome.

Due to concern for Alport syndrome and consideration of donor selection, the patient was referred for genetic counseling. Genetic testing of a commercial panel of 17 genes (Supplemental Table) identified a homozygous missense variant in the *COL4A4* gene: c.1180G>A (p.G394S). This glycine residue is highly conserved and present in a

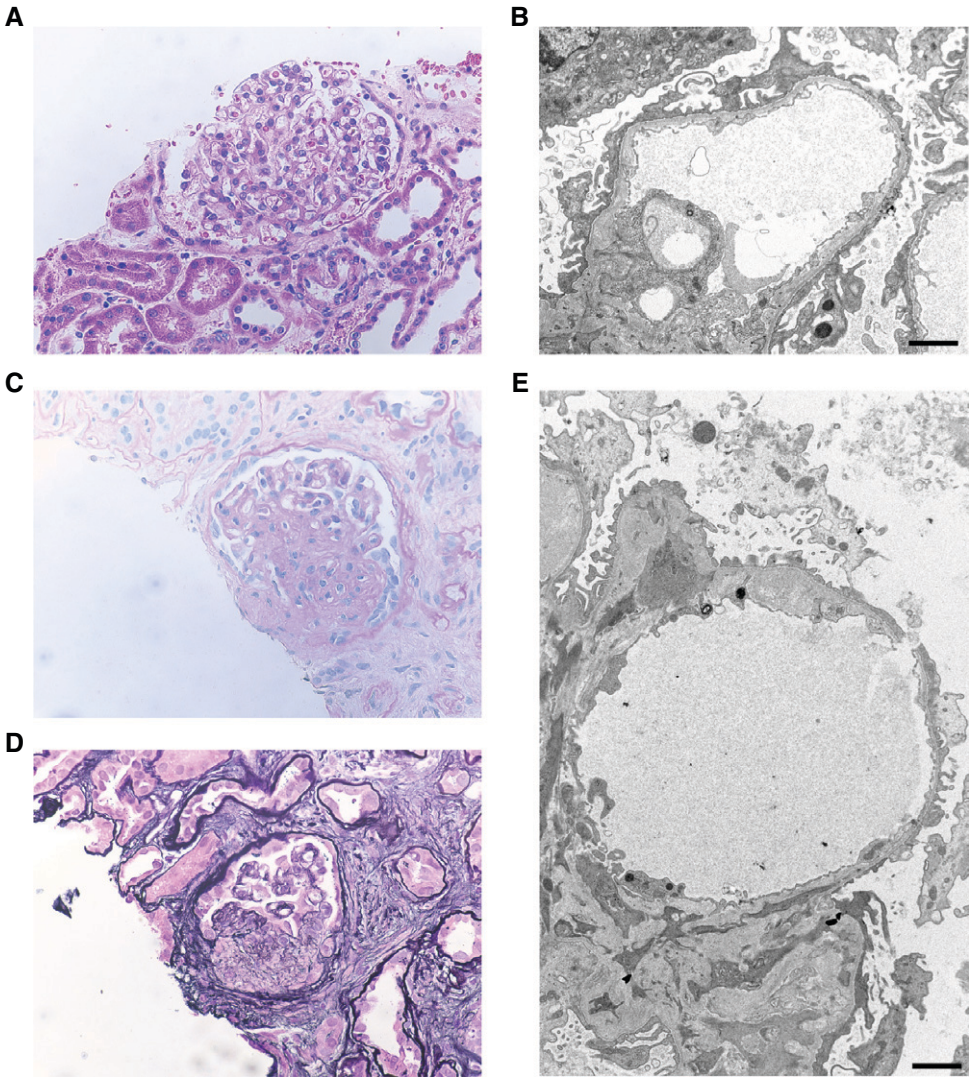

Figure 1. | **Histopathologic findings from the patient's first and second biopsies.** (A) Normal glomerulus sampled in the patient's initial biopsy (hematoxylin and eosin stain, ×400). (B) Ultrastructure of a glomerulus demonstrating significant thinning of the glomerular basement membrane (GBM), measuring approximately 160 nm. (C) and (D) Glomeruli with focal segmental glomerulosclerosis in the patient's second biopsy, approximately 13 years later (period acid–Schiff stain and Jones methenamine silver, respectively; ×400). (E) Ultrastructure of a glomerulus in the second biopsy demonstrates marked but segmental thinning of the GBM. Hallmark ultrastructural features of Alport syndrome, including multilamellation and "basket-weaving" of the GBM and "subepithelial scalloping," were not observed. Scale bars: 2 μm.

Gly-X-Y repeat. However, algorithms developed to predict the effect of missense changes on protein structure or function were not in agreement (SIFT- deleterious; PolyPhen-2- probably damaging; Align-GVGD- Class C0). Thus, this variant was classified as a VUS by the clinical laboratory. The variant is not present in a large population database (gnomAD; https://gnomad.broadinstitute.org/), indicating that it is rare (17). To our knowledge, the p.G394S variant had not been previously reported in association with disease. Genetic testing also identified a heterozygous pathogenic variant in the *PKHD1* gene, consistent with carrier status for autosomal recessive polycystic kidney disease, which is not expected to play a role in the patient's glomerular disease. Of note, the patient did not have kidney or liver cysts by abdominal imaging.

A heterozygous variant may falsely appear homozygous in the presence of a heterozygous deletion in *trans*. Thus, to detect a deletion in all or part of the *COL4A4* gene, we performed deletion/duplication analysis *via* oligo array comparative genomic hybridization. This analysis did not detect deletions involving the *COL4A4* or *COL4A3* genes, indicating that the p.G394S variant is likely present on both alleles in this patient.

Although a compelling candidate in the context of the patient's clinical presentation and biopsy findings, additional evidence supporting pathogenicity of the *COL4A4* p.G394S variant was needed before it could be established as diagnostic. Therefore, we performed functional evaluation of the *COL4A4* p.G394S variant to assess its pathogenicity further.

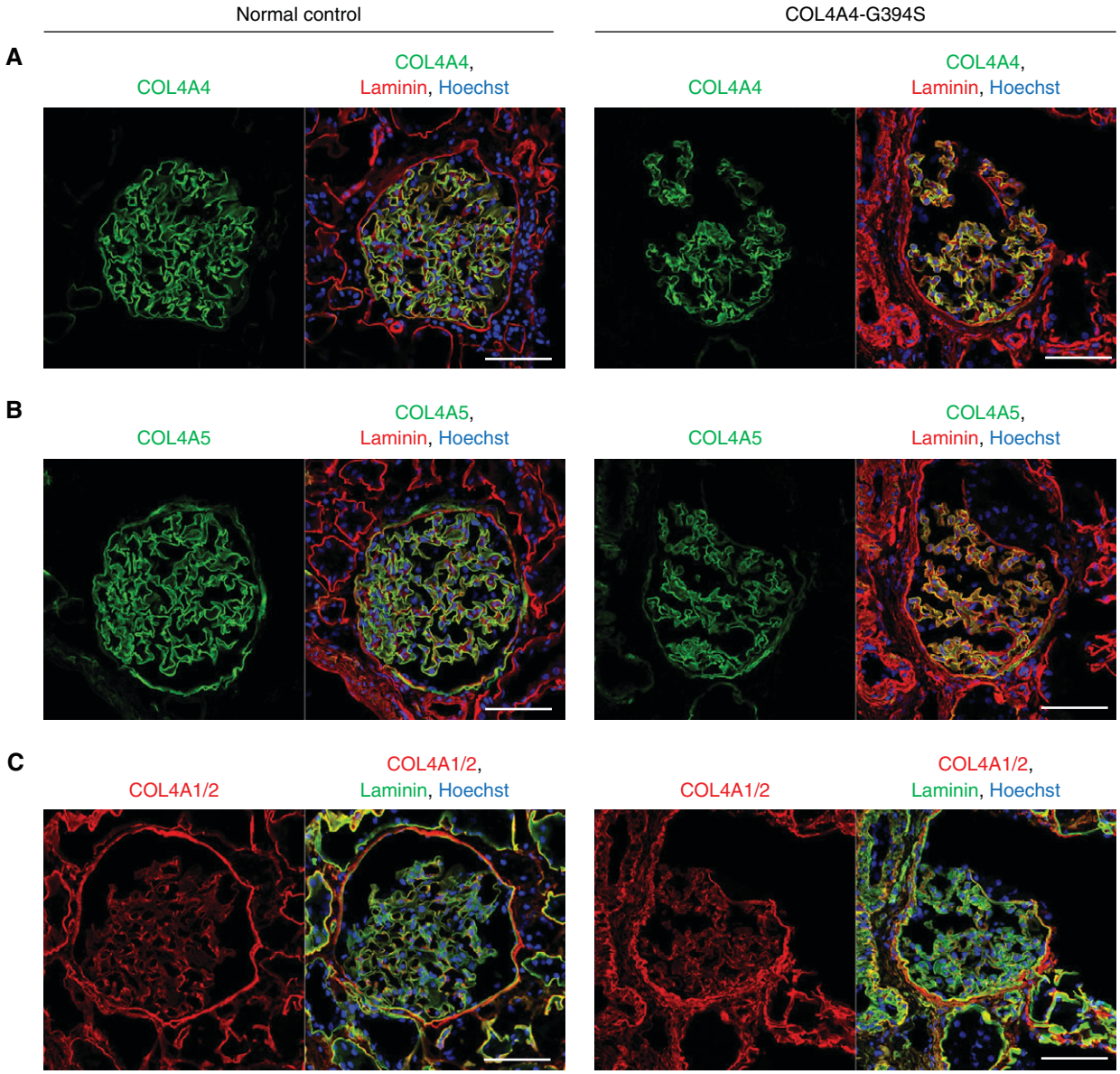

Figure 2. | Immunofluorescence staining for basement membrane components reveals a normal pattern in the patient's biopsy. (A–C) Frozen sections from a normal control kidney and from the patient's second biopsy were stained with the COL4A antibodies indicated along with anti-laminin-111 and Hoechst as basement membrane and nuclear counterstains, respectively. Both COL4A4 (A) and COL4A5 (B) are detected in the control and patient GBMs. (C) Antibody to COL4A1/2 show staining primarily in glomerular mesangium, Bowman's capsule, and tubular basement membranes in both control and patient sections. Scale bars: 100 μm.

The COL4A4-G394S Variant Impairs N-terminal Assembly and Secretion of the Collagen α3α4α5(IV) Heterotrimer

The split NanoLuc-based protein-protein interaction assay system is a method to detect close protein interactions by luminescence associated with the proximity of the split NanoLuc fragments LgBiT and SmBiT (18). Using this technology, we previously developed a method to evaluate the formation of proper collagen α3α4α5(IV) heterotrimers quantitatively (Figure 3A) (16, 19, 20). This split NanoLuc complementation-based α3α4α5(IV) assay has detected loss of function and presumed pathogenicity of many variants reported in patients with Alport syndrome.

To assay the function of the COL4A4-G394S variant, we used split NanoLuc assays in a defined workflow (Figure 3B) to compare it with WT and with two previously reported missense variants affecting conserved glycine residues: COL4A4-G545A as a nonpathogenic variant (21) and COL4A4-G960R as a pathogenic mutation that was found to be heterozygous in a patient with TBMN (22). In the C-terminal split NanoLuc tag assays, intracellular and extracellular luminescence levels for G394S and G545A were comparable to WT, but the extracellular luminescence level for the pathogenic G960R variant was 40% lower (Figure 3, C and D). On the other hand, in the N-terminal tag assays, the extracellular luminescence levels were 50% and 70% lower for G394S and

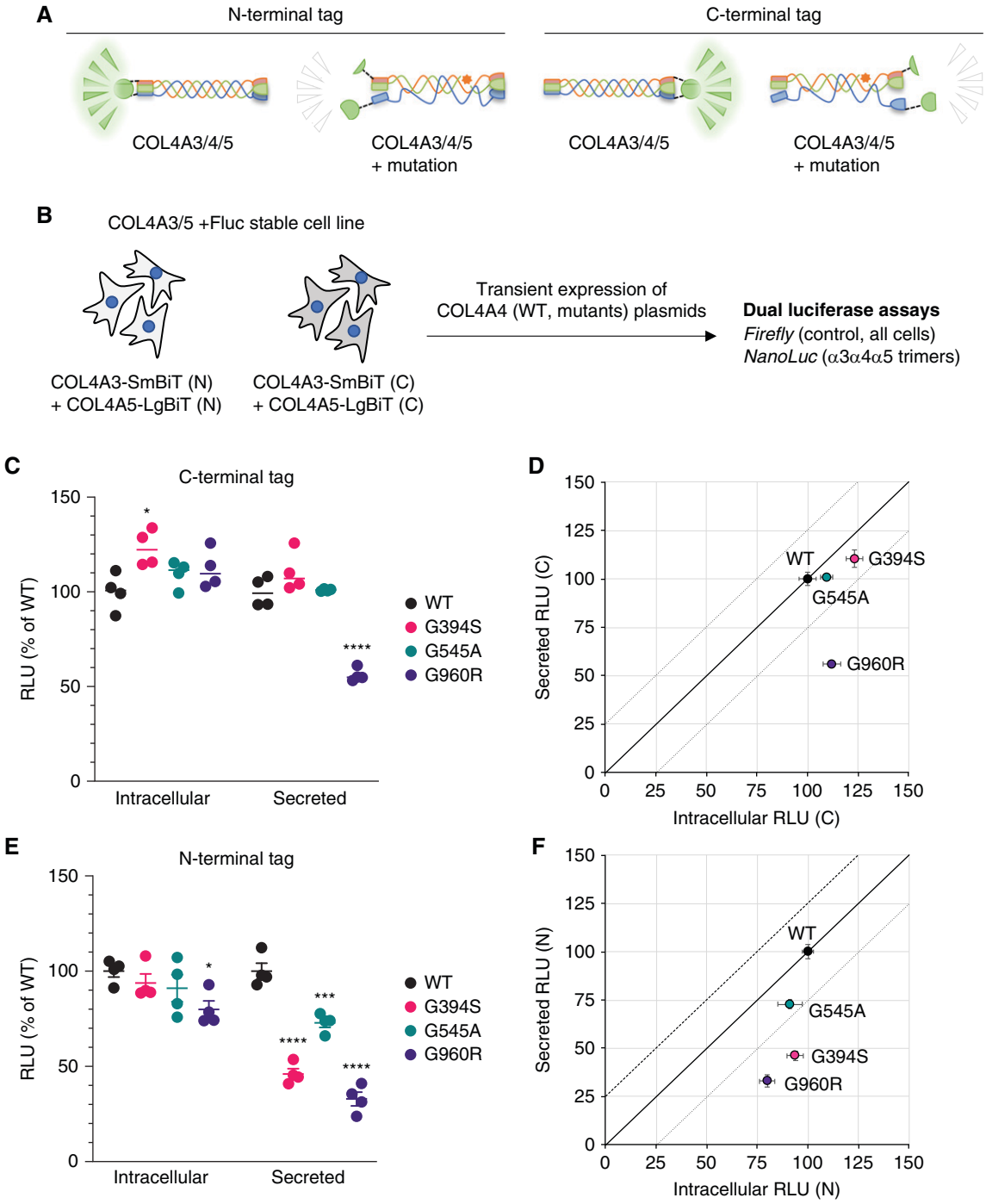

Figure 3. | **COL4A4 variants variably affect collagen α3α4α5(IV) heterotrimer formation and secretion.** (A) Schematic diagrams of the N-terminus (left) and C-terminus (right) split NanoLuc-based collagen α3α4α5(IV) trimer formation assays. (B) Schematic diagram of the experimental workflow to assay trimer assembly and secretion. (C and D) C-terminally tagged α3α4α5(IV) trimer formation assay. (C) Intracellular and secreted relative light units (RLU) from HEK293T cells expressing wild type (WT) COL4A4 or a variant. (D) Scatter plot of the intracellular/secreted RLU ratios. Solid line: Y=X. Dotted lines: Y=X+25, Y=X–25. (E and F) N-terminally tagged α3α4α5(IV) trimer formation assay. (E) Intracellular and secreted RLU from HEK293T cells expressing WT COL4A4 or a variant. (F) Scatter plot of the intracellular/secreted RLU ratios. Lines are as in (D). Data points below the Y=X–25 lines in (D) and (F) were considered to indicate a significant secretory defect. G960R, a known pathogenic variant, showed a secretory defect in both C-terminal and N-terminal α3α4α5(IV) assays, whereas G394S showed a defect only in the N-terminal α3α4α5(IV) assay. G545A is a known non-pathogenic variant. Error bars indicate the mean±SEM (n=4). *P<0.05; ***P<0.005; ****P<0.0001 versus WT (Dunnett's multiple comparison test).

G960R, respectively, and the nonpathogenic G545A variant exhibited slightly but significantly decreased luminescence (Figure 3, E and F). These results suggest that G394S impairs collagen α3α4α5(IV) trimer secretion by partially impairing N-terminal assembly.

Discussion

Using a combination of clinical, genetic, and functional evidence, we demonstrate that the COL4A4 p.G394S variant causes a mild form of autosomal recessive Alport syndrome. Establishing the etiology of kidney disease informs patient management and recurrence risks for family members. A diagnosis of Alport syndrome prompts ophthalmologic and otologic evaluations to assess for extrarenal manifestations of disease. Regarding family members, the patient's children are each expected to be heterozygous carriers of the p.G394S variant. Findings from the collagen assembly assay indicate the p.G394S variant causes a milder functional phenotype than other pathogenic variants. Of note, the patient has had long-standing diabetes mellitus and proteinuria. As we considered the risks/benefits of anticipated kidney transplantation, the current evaluation demonstrates absence of diabetic nephropathy and likely dysfunction of COL4A4. Thus, we would not anticipate recurrence of primary kidney disease as we typically would for diabetic nephropathy (23). We also suggest that family members who are heterozygous carriers for the p.G394S variant are unlikely to develop disease unless a second pathogenic variant is present in *trans*. However, there is an increased risk for autosomal recessive Alport syndrome in future generations.

VUS are a known challenge in clinical genetic testing. A variant is classified as a VUS when there is limited or conflicting evidence supporting its pathogenicity. The prevalence of VUS identified by genetic panels differs across disease areas and is influenced by the number of genes interrogated. Retrospective analyses of results from a small 17-gene and a large 382-gene kidney panel report that VUS were identified in 29% and 100% of individuals tested, respectively (24, 25). The presence of a VUS should generally not be used to inform patient management or risk assessment in family members, given the lack of clarity as to their true role in disease. Therefore, such findings are considered uninformative, and patient management is instead informed by personal and family history. With new evidence, although variants may be reclassified over time, there are no widely accepted guidelines as to how frequently, or by whom, variant reassessment should occur.

In an era of precision medicine, translational science approaches to determine pathogenicity of VUS are needed. Functional assays are available for certain genes; however, these largely exist in research settings, which are typically low throughput and rely on grant funding to answer specific research questions. Additionally, there is a dearth of clinically valid protocols for integrating functional assessment into clinical care. However, as demonstrated by this work, Alport syndrome is well suited for translational science approaches because there are multiple functional assays available to ascertain *COL4A* variant pathogenicity, including evaluation of trimerization and secretion and electrophoresis and thermal stability assays (4). This case also highlights the limited diagnostic sensitivity of immunofluorescence studies, reinforcing the concept that a normal collagen α3α4α5(IV) staining pattern does not rule out the diagnosis of Alport syndrome.

The trimerization of type IV collagen α chains is initiated *via* association of the three C-terminal NC1 domains, then the three collagenous domains "zipper up" toward the N-terminus, which forms the trimeric 7S domain. Therefore, mutations in the NC1 domain that impair NC1 domain trimerization often strongly disrupt trimer secretion, and thus they are pathogenic. However, mutations such as G394S that do not affect NC1 domain assembly but inhibit 7S domain assembly and secretion of trimers can also be pathogenic. Our data (Figure 3, E and F) showed that both the known pathogenic mutation G960R and the new G394S variant decreased extracellular luminescence, reflecting reduced secretion of properly assembled α3α4α5(IV) heterotrimers that causes the resulting phenotypes (Figure 4). On the other hand, neither variant reduced intracellular luminescence, reflecting either efficient intracellular α3α4α5(IV) heterotrimer formation or buildup of assembled trimers that are not efficiently secreted. Discrepancies between intracellular and extracellular luminescence have been observed for many pathogenic *COL4A5* missense mutations (16). Both our data and previous data suggest that some mutations that impair heterotrimer secretion can cause formation of incomplete complexes within the cell, but these complexes are dissociated during intracellular trafficking toward secretion. The mechanism by which incomplete complexes dissociate is not clear. Molecular chaperones and the pH are different in the endoplasmic reticulum, where collagen IV heterotrimers are assembled, and in the Golgi, where heterotrimers are subsequently modified. These aspects of protein maturation may be involved.

Mutations that affect only N-terminal assembly can cause mild Alport syndrome–like presentations or TBMN. Indeed, there is evidence that glycine substitutions near the N-terminus are in general less pathogenic than those near the C-terminus (26). The exact correlation between the degree of reduction in α3α4α5(IV) heterotrimer trimer formation and/or secretion and the development of Alport syndrome and TBMN is difficult to elucidate on the basis of the results of this study alone. However, the accumulation and analysis of similar cases in the future should make it possible to define criteria for evaluating the pathogenicity of novel variants.

In conclusion, we utilized genetic sequencing and a collagen assembly assay to reclassify a novel VUS as mildly pathogenic. The results provide clinically actionable data for the patient and her family and provide a blueprint to understand the molecular consequences of this and similar variants better. High-throughput approaches have highlighted the importance of mutations in *COL4A3*, *COL4A4*, and *COL4A5* for the pathogenesis of human kidney disease, and low-throughput approaches, applied broadly, may provide a critical next step for precision medicine. This study further demonstrates the importance of collaborations between clinicians and basic scientists for research in Alport syndrome (27).

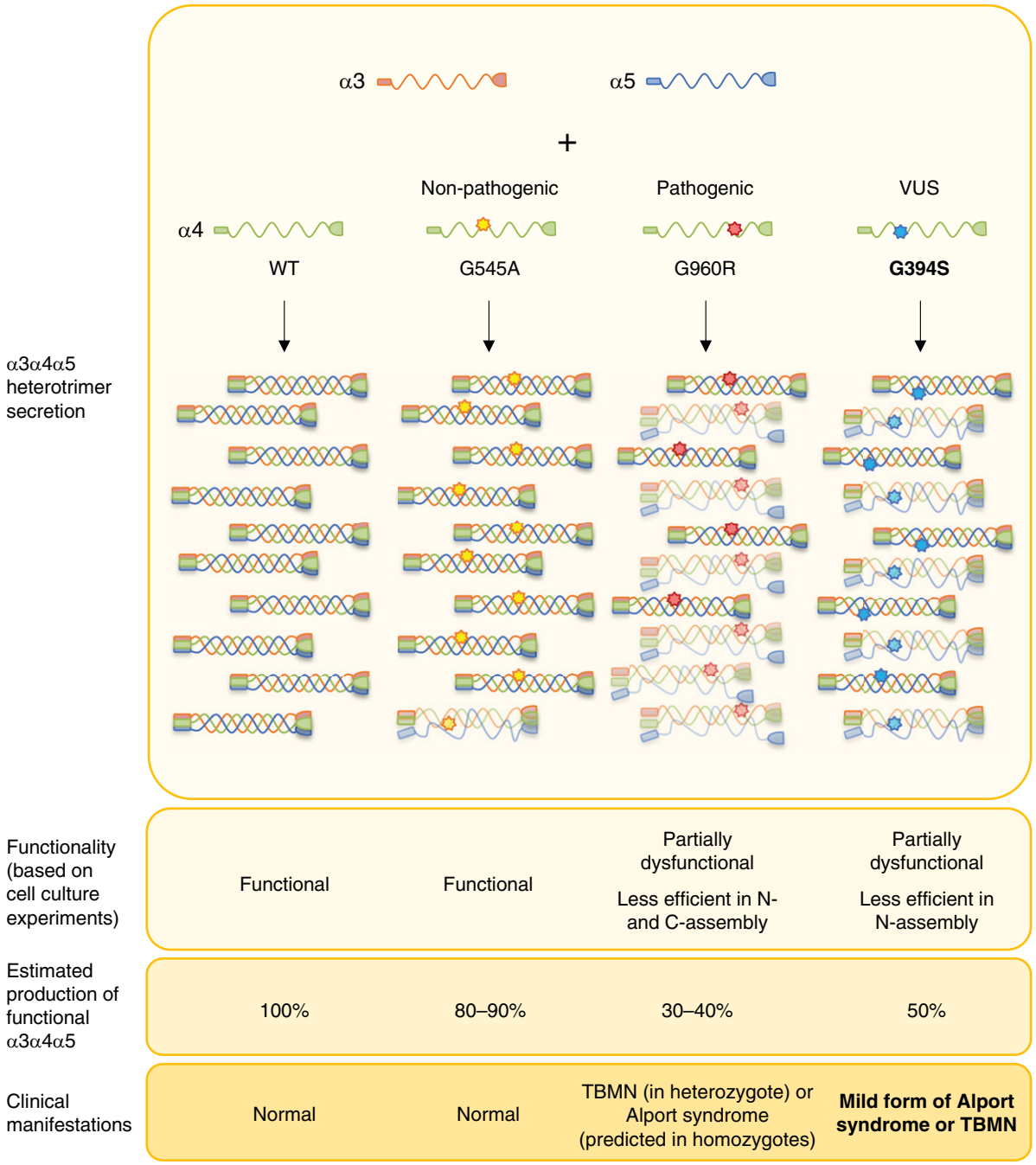

Figure 4. | **Schematic depiction of how the variant collagen α4(IV) chains assayed here impair trimer formation and secretion to varying degrees.** The degree of successful trimerization and secretion determines the functionality of the collagen α3α4α5(IV) network and the resulting clinical manifestations.

Disclosures

V. Bhalla reports consultancy for Bayer Pharmaceuticals, CareDx, Guidepoint LLC, Janssen Pharmaceuticals, LEK Consulting, and Reata; ownership interest in Pyrames Health and Viscira LLC; research funding from CareDx (spouse); honoraria from Bayer Pharmaceuticals and CareDx (spouse); an advisory or leadership role for the *American Journal of Physiology—Renal Physiology* (editorial board; July 2007–present), CareDx (scientific advisory board; spouse), *Physiologic Reports* (April 2018–present), Pyrames (scientific advisory board), and Relypsa (scientific advisory board); participation in a speakers' bureau for CareDx (spouse); and other interests or relationships with the American Medical Association, Validated Device Listing-Blood Pressure Measurement Devices, Hypertension Council, American Heart Association (member), and Kidney and Cardiovascular Disease Council, American Heart Association (immediate past-chair). J.H. Miner reports consultancy for Alpha Insights, AstraZeneca, Bridge Bio, Deerfield Management, Guidepoint, Janssen Biotech, Inc., Mantra Bio, National Institutes of Health, and The Planning Shop; research funding from Cadre/Myonid Therapeutics, Chinook Therapeutics, and Lung

Therapeutics, Inc.; honoraria from Pfizer; patents or royalties from Angion, Genentech, Kerafast, and Maze Therapeutics; an advisory or leadership role for the American Society for Matrix Biology (president), *Journal of Clinical Investigation* (consulting editor), *Kidney International* (editorial board), *Matrix Biology* (editorial board), *Matrix Biology Plus* (editorial board); and other interests or relationships with the Alport Syndrome Foundation (scientific advisory research network). All remaining authors have nothing to disclose.

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Author Contributions

V. Bhalla, V. Charu, J.H. Miner, and K. Omachi conceptualized the study; V. Charu, J. Kohler, and K. Omachi were responsible for the formal analysis; J.H. Miner was responsible for funding acquisition and supervision; J.H. Miner and K. Omachi were responsible for the methodology; K. Omachi was responsible for data curation; and all authors were responsible for the investigation, wrote the original draft of the manuscript, reviewed and edited the manuscript, and approved of the final manuscript.

Data Sharing Statement

All data are included in the manuscript and/or supporting information.

Supplemental Material

This article contains the following supplemental material online at http://kidney360.asnjournals.org/lookup/suppl/doi:10.34067/KID.0005472022/-/DCSupplemental.

Supplemental Table. Seventeen genes tested as part of commercial panel.

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