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Rare hereditary COL4A3/COL4A4 variants may be mistaken for familial focal segmental glomerulosclerosis

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Abstract

Focal segmental glomerulosclerosis (FSGS) is a histological lesion with many causes including inherited genetic defects with significant proteinuria being the predominant clinical finding at presentation. Mutations in *COL4A3* and *COL4A4* are known to cause Alport syndrome, thin basement membrane nephropathy, and to result in pathognomonic glomerular basement membrane findings. Secondary FSGS is known to develop in classic Alport Syndrome at later stages of the disease. Here, we present seven families with rare or novel variants in *COL4A3* or *COL4A4* (six with single and one with two heterozygous variants) from a cohort of 70 families with a diagnosis of hereditary FSGS. The predominant clinical findings at diagnosis were proteinuria associated with hematuria. In all seven families, there were individuals with nephrotic range proteinuria with histologic features of FSGS by light microscopy. In one family, electron microscopy showed thin glomerular basement membrane, but four other families had variable findings inconsistent with classical Alport nephritis. There was no recurrence of disease after kidney transplantation.

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Families with *COL4A3* and *COL4A4* variants that segregated with disease represent 10% of our cohort. Thus, *COL4A3* and *COL4A4* variants should be considered in the interpretation of next-generation sequencing data from such patients. Furthermore, this study illustrates the power of molecular genetic diagnostics in the clarification of renal phenotypes.

Keywords

focal segmental glomerulosclerosis; podocyte; proteinuria; Alport syndrome

Introduction

Focal segmental glomerulosclerosis (FSGS) is the third leading cause of end-stage kidney disease (ESKD) in the USA and its incidence is estimated at 23 per million population[1]. Current treatments for primary FSGS frequently fail to induce remission and are poorly tolerated[2,3]. Proteinuria is frequently the earliest clinical manifestation of FSGS and can predate the development of renal failure by months or years[4]. A number of podocyte-specific gene mutations have been shown to cause familial FSGS including *TRPC6, ACTN4, WT1, CD2AP, INF2, NPHS1, NPHS2* and *PLCE1*[5-12]. This enrichment of single gene causes of FSGS in the podocyte suggests that this cell plays a central role in the pathogenesis of proteinuria and FSGS. However, the podocyte is part of a complex glomerular filtration barrier that is responsible for the permselectivity of the glomerulus. Defects in the glomerular basement membrane (GBM) can cause Alport Syndrome (AS) and thin basement membrane nephropathy (TBMN). AS consists of a triad of progressive renal failure, hearing loss and ocular abnormalities and occurs due to mutations in *COL4A5* in 85% of cases[13]. Classic X-linked disease presents in childhood with microscopic or gross hematuria and progression to ESKD in the second to third decade of life[13]. Autosomal dominant and autosomal recessive AS due to mutations in *COL4A3* and *COL4A4* genes are less common and their phenotype is more variable when compared with X-linked disease[14-17]. One of the variable clinical manifestations that have been reported in cohorts of patients with autosomal AS is the presence of proteinuria and changes consistent with FSGS on kidney biopsy. These changes often occur late in the course of the disease and are typically reported as secondary changes due to the primary glomerular basement membrane defect induced by abnormal collagen. It is therefore conceivable that some patients with collagen(IV) related kidney disease may phenocopy both idiopathic and familial FSGS. To the best of our knowledge, there are no studies looking at the prevalence of rare variants in *COL4A3* and *COL4A4* in a cohort of patients with a diagnosis of familial FSGS.

In this study, we performed whole-exome sequencing (WES), podocyte-exome sequencing (PES) or direct sequencing on 70 families with a diagnosis of familial FSGS. We found that 7 out of 70 families (10%) in our cohort have rare variants in *COL4A3* and *COL4A4*. Based on these findings we posit that rare variants in *COL4A3* and *COL4A4* are common in a cohort of patients with familial FSGS and some of these variants may be disease causing. Furthermore, our findings illustrate the role of molecular diagnosis in accurate disease classification.

Results

We identified 70 families with familial FSGS of unknown cause. This cohort included our index family, Family DUK6696.

Index kindred; Family DUK6696

Three female siblings presented with nephrotic range proteinuria and hematuria between 8 and 12 years of age. The oldest sibling had a biopsy at diagnosis, which showed classical features of FSGS (Figure 1). She progressed to ESKD within four years. No affected individuals have been transplanted (Table 1). The two parents are well and are not known to have any kidney disease. The referring physician made a diagnosis of familial FSGS and the family was referred to our group for genetic studies. Whole exome sequencing (WES) was performed on this family and analyzed using our filtering algorithm as described in supplementary Figure 1. We did not find any disease causing mutations in any known FSGS genes, however; we identified a novel compound heterozygous truncating variants in trans (E131Xfs151 and Q936X) in *COL4A3* (Figure 2). These two novel variants were the only two variants that segregated with disease in this family.

Analysis of a familial FSGS cohort for COL4A3 and COL4A4 variants

Based on these findings, a directed search for rare variants in *COL4A3* and *COL4A4* was undertaken in 62 additional families that were referred to us with familial FSGS using next generation sequencing (NGS: WES and PES). Furthermore, we performed direct sequencing of all the exons and the exon/intron borders in *COL4A3* and *COL4A4* in seven families that did not have NGS data. We identified an additional six families with rare or novel *COL4A3* or *COL4A4* variants that segregated with disease. Thus, seven families out of 70 (10%) in this cohort were found to have rare or novel *COL4A3* or *COL4A4* variants. Of these, only the index family had a compound heterozygous variant, the other six families had a single heterozygous variant. The phenotypes of these seven families are shown in Table 1. The biopsy diagnosis of FSGS was made based on 1) the presence of focal, segmental areas of glomerular sclerosis, often with associated hyalinosis and adhesions of sclerotic tufts to Bowman's capsule, 2) the presence of some degree of podocyte foot process effacement as assessed by electron microscopy, and 3) the absence of immune deposits or other defined causes of glomerulonephritis as assessed by immunofluorescence or electron microscopy. Specific immunofluorescent staining for alpha chains of type IV collagen was not performed. A representative biopsy from one of these seven families, Family DUK6534, is shown in Figure 3. Light microscopy (LM) showed segmental sclerosis with hyalinosis and adhesion to Bowman's capsule. Electron microscopy (EM) showed localized effacement of podocyte foot processes and localized areas of basement membrane denudation with loss of podocyte cytoplasm. Glomerular basement membranes are slightly irregular but they have normal thickness with no lamination. Supplementary Figure 2 shows pedigrees for the additional six families.

Genotype phenotype correlation

There are three families (Families DUK6534, DUK6630 and DUK6527) with missense variants at glycine residues in the collagenous domain of α3 or α4 collagen(IV), two

families (Families DUK6531 and DUK6585) with missense variants in the NC1 domains and two families (Families DUK6696 and DUK6669) with truncating variants in *COL4A3* and *COL4A4* (Table 2). There is intra-family and inter-family variation in phenotype among the seven families (Table 1). However, at least one individual in each of the three Families, DUK6696, DUK6585 and DUK6669 presented with nephrotic range proteinuria and/or FSGS on renal biopsy in the first two decades of life. The family with the compound heterozygous truncating variant had early onset disease with nephrotic range proteinuria compared with the families with single heterozygous variants. The families with missense mutations in the NC1 domain of α3 collagen(IV) had no obvious difference in phenotype or histology compared to the families with glycine variants. Electron microscopy (EM) data was available in five of the seven families with collagen (IV) variants (Families DUK6534, DUK6585, DUK6527, DUK6630 and DUK6669). Thin glomerular basement membrane (GBM) was found in Family DUK6630 with an average thickness of 230nm. In Families DUK6527, DUK6585, and DUK6669 the GBMs were thickened and wrinkled. In one family, Family DUK6534, the GBM thickness and architecture were normal. Basement membrane splitting, lamellation and basket weaving typical of AS were absent in all of the EM images evaluated. In those individuals who received a kidney transplant there was no recurrence of disease. The only obvious phenotypic difference between the families with *COL4A4* variants (Families DUK6527, DUK6669) and those with *COL4A3* variants was the presence of hearing loss in the *COL4A3* families. In all the families with hearing loss, history of hearing loss was established post priori after *COL4A3* variants were identified.

COL4A3 and COL4A4 variant locations

The *COL4A3* and *COL4A4* variants did not cluster in any particular region of either gene. Specifically, no variants lie in the KRGDS motif in the α3 chain. This motif in the α3 chain of collagen(IV) has been shown to bind to integrin proteins on human podocytes *in vitro*[18]. Missense variants were present in the collagenous domains and NC1 domains of *COL4A3* and *COL4A4*. The truncating variants were found at different locations within the collagenous domains of *COL4A3* and *COL4A4* (Figure 4).

Search for modifier genes

To explain the prominent podocyte related features in the seven families, we searched for rare heterozygous and homozygous variants in *NPHS1 and NPHS2*, we did not find any variants in these genes in any of the seven families.

Discussion

This is the first report of *COL4A3* and *COL4A4* variants in a cohort of patients with a primary diagnosis of familial FSGS[19]. Previous studies have reported findings of FSGS in cohort of patients with collagen(IV) related kidney diseases, however the biological significance of these findings is unknown. Controversy exists as to whether FSGS in the context of collagen(IV) related kidney disease is a primary process or whether it occurs secondary to the GBM pathology[20-23]. This distinction is not clear-cut in all renal diseases associated with collagen(IV) abnormalities given their variable natural history and phenotypes. The findings from the present study seem to suggest that mutations in mature

GBM collagen(IV) may play a direct role in the pathogenesis of FSGS and that the phenotypes induced by mutations in mature GBM collagen(IV) genes may phenocopy primary FSGS.

The patients with *COL4A3* and *COL4A4* variants described in our study had significant proteinuria with hematuria at diagnosis. This is in contrast with AS where proteinuria and secondary FSGS tend to occur late in the course of the disease[14]. Diagnostic biopsies in our families had the typical findings of focal segmental sclerosis on LM and foot process effacement on EM but GBM morphology was not typical of AS. These findings of a mixed phenotype characterized by chronic progressive glomerular disease, hematuria and significant proteinuria at diagnosis suggest that *COL4A3* and *COL4A4* mutations can cause a wide spectrum of disease phenotypes from AS to FSGS.

The *COL4A3* mutation in Family DUK6696 is most likely disease causing. Analysis of WES data in this family showed that the truncating compound heterozygous variant in *COL4A3* is the only variant segregating with disease in this family. In the set of seven families, three of the segregating variants from two families are novel (not in dbSNP, 1000 Genomes or NHLBI Exome Variant database). The variants described in five of the seven families are listed in the NHLBI Exome Variant Server (EVS) database however; these variants are found at very low frequencies of between 0.003 and 0.000085 in over 11,000 chromosomes. Furthermore, four of these five variants have been previously associated with disease and are curated in the Leiden Open Variation Database (https://granada.lumc.nl/LOVD2/). These data make it more plausible that these variants can cause disease. In addition, all the variants were predicted to be damaging by three independent *in-silico* software programs (polyphen, SIFT, mutation taster) and were all conserved in evolution, suggesting that they are likely to be pathogenic.

How mutations in mature GBM collagen might cause proteinuria is not understood. Altered permselectivity of the GBM, abnormal matrix-podocyte interactions and defective expression or trafficking of GBM matrix components by the podocyte are all possible mechanisms of disease. It is known that mutations in *LAMB2* can cause FSGS[24,25]. This gene encodes for laminin β2, part of one major GBM protein, Laminin-521. Defective interaction of laminin-521 with podocyte integrin proteins is suggested as a possible mechanism of disease [24]. It has also been demonstrated that α3 collagen(IV) can bind to integrin proteins on human podocytes via a KRGDS motif *in vitro*[18]. In our study, there were no consistent GBM ultrastructural findings in the families with *COL4A3* and *COL4A4* variants. Conversely, podocyte foot process effacement was a constant finding and suggests that the observed phenotype may be due to podocyte abnormalities. Availability of data showing reduced staining of collagen (IV) in the glomerular basement membrane would have provided supportive evidence that the phenotype observed in this cohort of patients are due to rare variants in *COL4A3* and *COL4A4* genes, unfortunately this was not possible because of our inability to access suitable tissue for immunostaining. The previously described *COL4A3* and *COL4A4* variants found in AS and TBMN cohorts are evenly spread throughout the coding region of these genes. This is also the case for the collagen variants described in our cohort. Thus, there appears to be no particular domain or region of the α3 or α4 proteins that might confer podocytopathy like disease expression.

It is possible that the variable phenotypes found in this study could be due to variants in FSGS genes acting as disease modifiers for collagen(IV) related kidney disease. However, we did not find novel or known variants in genes known to cause FSGS in this cohort. In the families analyzed with NGS methods, we were unable to rule out other variants that may be pathogenic because of small pedigree size. It is therefore possible that there are yet to be identified podocyte/FSGS genes that may be acting as modifiers. Evidence in support of this is a study by Korstanje *et al.* that showed the degree of proteinuria in mice with a *COL4A4* mutation is dependent on their genetic background[26]. Conversely, it is possible that some of these families have mutations in currently unknown FSGS genes and *COL4A3* or *COL4A4* may be functioning as modifier genes.

The clinical significance of our findings is that there is an overlap between phenotypes induced by *COL4A3* and *COL4A4* variants and familial FSGS genes; therefore screening for rare variants/mutations in these genes in families referred with a diagnosis of familial FSGS is justified for better disease definition and treatment. Furthermore exclusion of variants in these genes should be thoughtfully contemplated as part of a filtering algorithm in the analysis of WES data in familial FSGS. In the present study, WES, using a standard library preparation, resulted in an average coverage depth of over 60X for both genes and for *COL4A5* (Supplementary Table 1). Current commercial testing of the coding regions of *COL4A3, COL4A4* and *COL4A5* by direct Sanger sequencing is more expensive than commercial WES by almost a factor of 10. Therefore with the development of robust and efficient bioinformatics pipelines for the analysis of WES data, NGS may represent a less expensive method to diagnose collagen(IV) related kidney disease. Finally this study emphasizes the role molecular diagnosis can play in aiding the phenotypic characterization of different kidney diseases and selection of appropriate treatment modalities.

Materials and Methods

Institutional Review Board approval was obtained from Duke University Medical Center (Durham, NC).

Clinical data

Inclusion criteria and determination of affection status are as previously reported[27]. Briefly, inclusion in this analysis required at least one individual with biopsy-proven FSGS and a second family member with biopsy-proven FSGS and/or ESKD. Clinical evaluation of these kindreds included a full family history, physical examination, urinalysis with qualitative or quantitative proteinuria and serum creatinine assay when appropriate. The biopsy diagnosis of FSGS was made based on 1) the presence of focal, and segmental areas of glomerular sclerosis, 2) the presence of some degree of podocyte foot process effacement as assessed by electron microscopy, and 3) the absence of immune deposits or other defined causes of glomerulonephritis as assessed by immunofluorescence or electron microscopy. Renal pathology reports and slides were reviewed when available for affected individuals. We also recorded the presence or absence of any extra renal manifestations such as hearing loss and ocular defects a priori and post priori.

Whole-exome sequencing and Podocyte-exome sequencing

Whole-exome sequencing was performed on 25 families. The Illumina TruSeq kit was used to create a 62Mb target region exome library and a customized sequence variant analyzer software was used[28]. Podocyte-exome sequencing was performed on 38 families. An exon capture sequence chip containing 2,400 genes that are enriched in the podocyte was designed by Dr Andrey Shaw (Washington University, St Louis, MO). The gene list was derived from microarrays of human and mouse podocyte cell lines and glomeruli from human kidney biopsies. All known FSGS or chronic kidney disease genes identified in genome-wide association studies were added to the list. MetaCore function of GeneGO (GeneGo.com) was used to place all of these genes into pathways. Sequencing used a next generation platform and variants were called and annotated using a standardized pipeline. We used our standard filtering algorithm (supplementary figure 1) to identify the disease causing variants in the index family (Family 6696).

Direct sequencing

Genomic DNA was extracted from whole blood using the Qiagen/Puregene kit (Qiagen, Hilden, Germany). Mutation analysis was carried out by sequencing of both strands of all exons of *COL4A3* and *COL4A4* using exon-flanking primers; primer sequences are listed in supplementary tables 2 and 3. All sequences were analyzed with the Sequencher software (Gene Codes, Ann Arbor, MI).

Seven families were subjected to direct sequencing using exon primers for *COL4A3* and *COL4A4* (In total 70 families were studied, 25 with WES, 38 with PES and 7 with Sanger sequencing).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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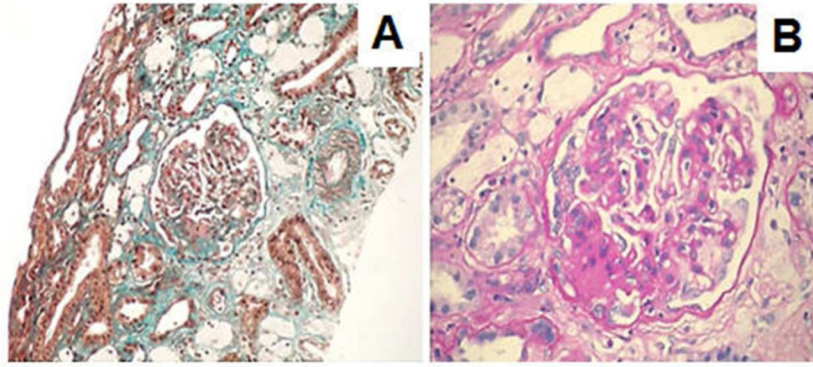
Figure 1. Family DUK6696 proband biopsy

(A) Masson's trichrome stain at 10X. There is segmental glomerular sclerosis in the hilar region with moderate interstitial fibrosis, clusters of foam cells in the interstitium, and tubular atrophy. (B) Periodic acid-Schiff stain of tissue from same individual.
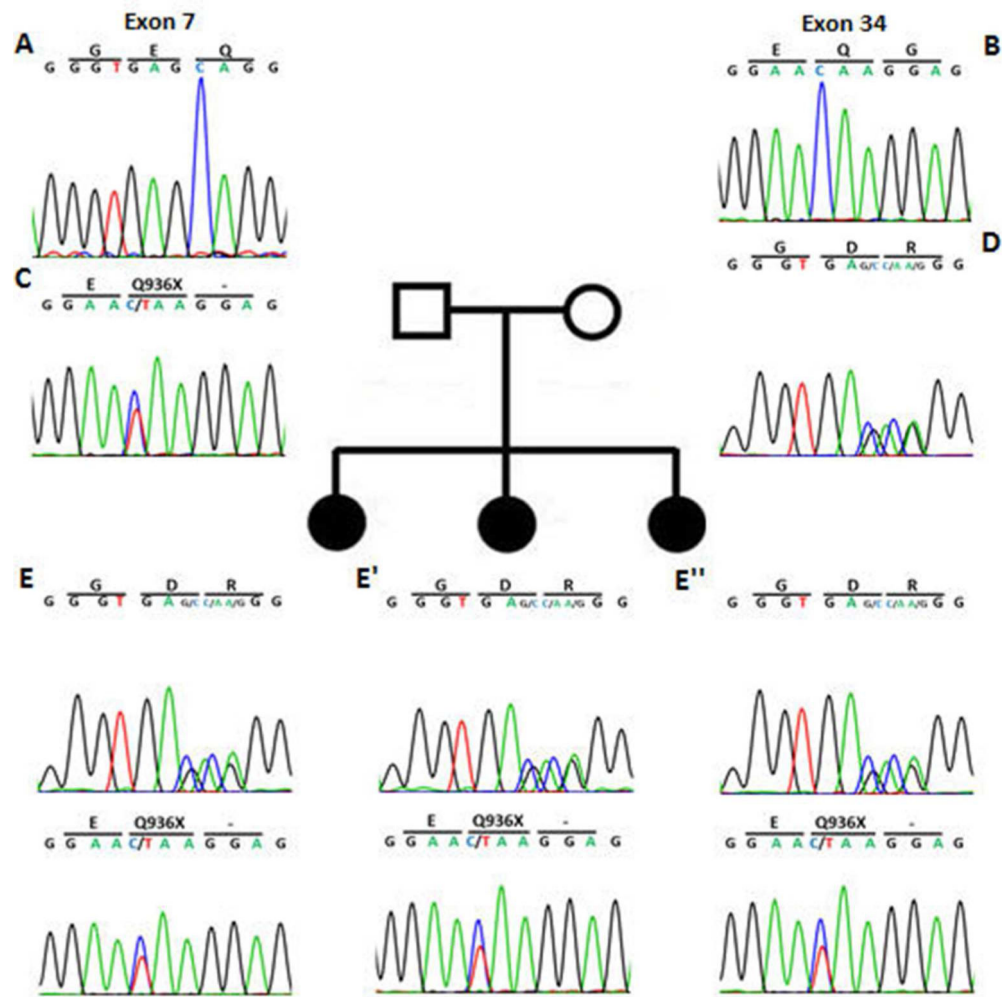
Figure 2. *COL4A3* variants in Family DUK6696
(A) Reference chromatogram in exon 7. (B) Reference chromatogram in exon 34. (C) Chromatogram of heterozygous E131fsX151 variant in father, exon 7. (D) Chromatogram of heterozygous Q936X variant in mother, exon 34. (E, E', E'') Chromatograms of the three affected siblings showing E131fsX151 and Q936X compound heterozygous variants *in trans*.
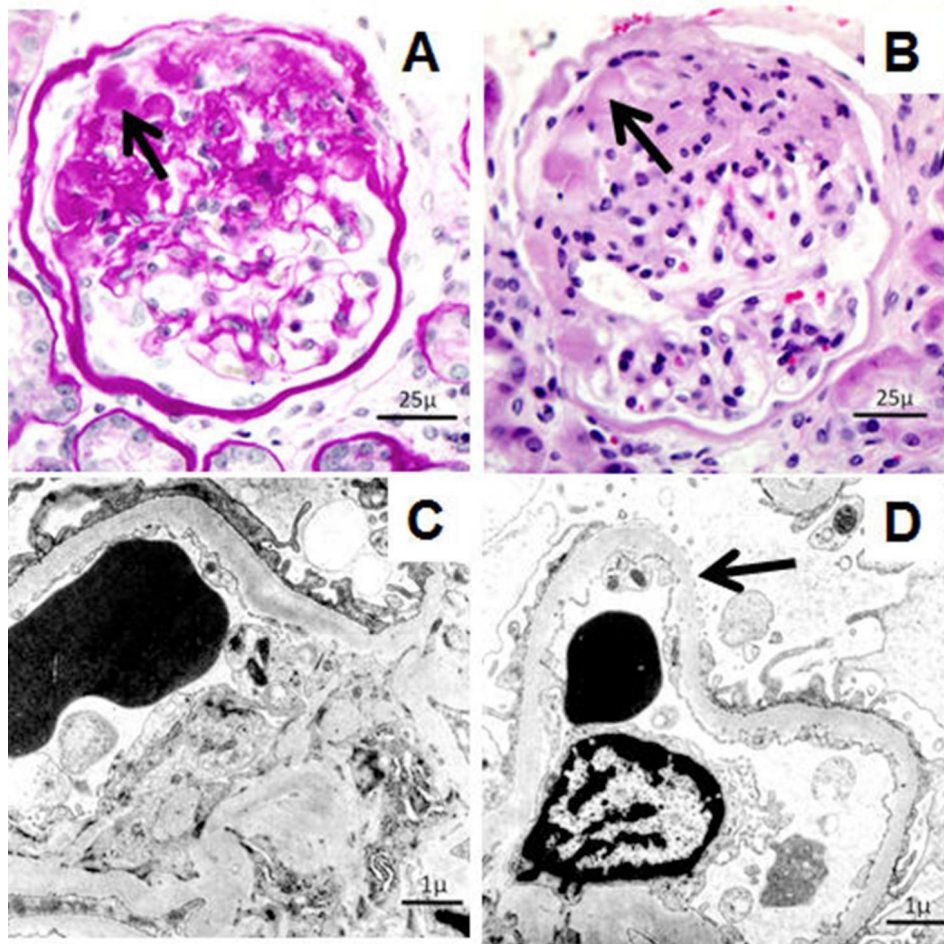
Figure 3. Family DUK6534 proband biopsy
A and B) The same glomerulus stained with periodic acid-Schiff (A) and hematoxylin and eosin (B) showing segmental sclerosis with hyalinosis (arrows) and adhesion to Bowman capsule. C and D) Electron microscopy from the same individual showing two different capillary loops. Both have localized effacement of podocyte foot processes. Area of basement membrane denudation with loss of podocyte cytoplasm (arrow). Glomerular basement membranes are slightly irregular but are of normal thickness with no lamination.
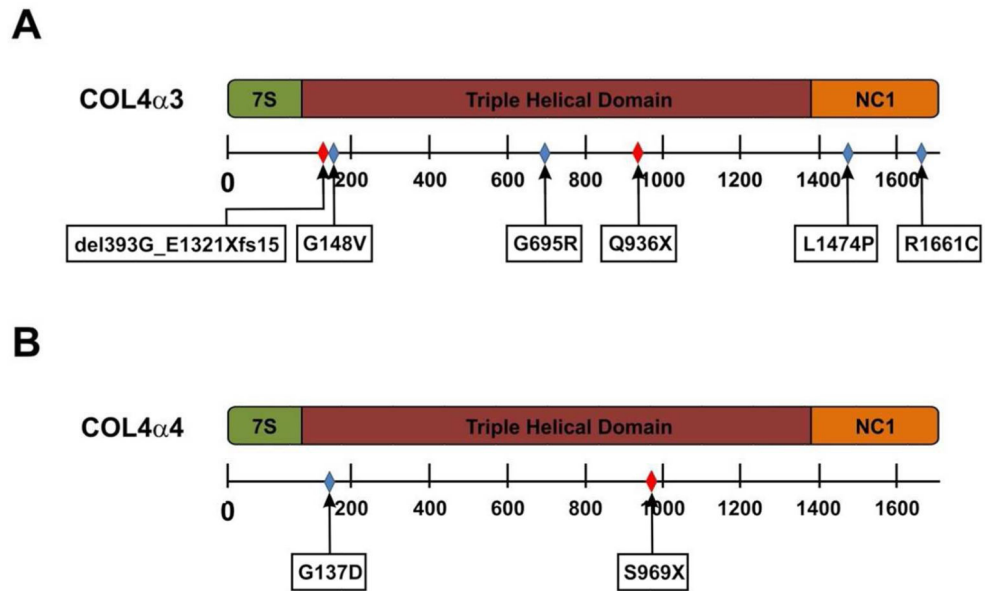
Figure 4. *COL4A3* and *COL4A4* variants relative locations
(A) Cartoon of the α3 collagen(IV) protein illustrating the locations of variants described in this study. (B) Cartoon of the α4 collagen(IV) protein illustrating the locations of variants described in this study. "7S" and "NC1" refer to the canonical cysteine-rich, 25-amino acid N-terminal domain and the C-terminal globular non-collagenous domains respectively which flank the central triple helical domain. Blue diamonds represent missense variants and red diamonds represent frame shift and nonsense variants.

Table 1

Phenotype information for seven families with *COL4A3* or *COL4A4* variants.

Family	Ethnicity	Individual	Gender	Age at Diagnosis	Hematuria	Proteinuria	Time to ESKD yrs	Biopsy findings LM	Biopsy findings EM	Transplant/Recurrence	Hearing loss/Ocular disease
DUK6696	Cau	1	F	8	Y	3.5g/d	4	FSGS	No EM	N	Y/N[#]
		100	F	13	Y	6.5g/d	U	U	U	N	Y/N[#]
		101	F	8	Y	2g/d	U	U	U	N	N
DUK6531	Cau	1	M	36	U	U	4	FSGS	No EM	Y/N	N
		101	M	36	U	U	4	FSGS	No EM	Y/N	N
DUK6534	Cau	1	F	35	Y	3.5g/d	U	FSGS	GBM normal thickness FP effacement	N	N/N
		1000	M	65	U	U	17	U	U	N	N/N
DUK6585	Cau	1	M	18	Y	3+	19	FSGS	GBM thickened FP effacement	Y/N	N/N
		101	M	33	U	5g/d	U	FSGS	U	N	N/N
		1000	M	U	U	2+	U	U	U	N	Y/N[#]
DUK6630	Cau	1	F	36	Y	2.3g/d	24	FSGS	Thin GBM, 230nm No lamellations FP effacement	Y/N	Y/N[#]
		103	F	64	N	1.7g/d	U	U	U	N	Y/N[#]
		9000	M	39	Y	1+	U	U	U	N	Y/N[#]
		9002	F	37	U	4+	U	FSGS	Thin GBM, 200nm FP effacement	N	Y/N[#]
		9003	M	40	Y	Neg	U	U	U	N	Y/N[#]
DUK6527	AA	1	F	35	Y	4g/d	U	FSGS	GBM Thickened GBM wrinkling FP effacement	N	N
		113	M	32	NA	2+	10	U	U	N	N
		1001	F	32	NA	3g/d	9	FSGS	GBM Thickened	N	N

Family	Ethnicity	Individual	Gender	Age at Diagnosis	Hematuria	Proteinuria	Time to ESKD yrs	Biopsy findings LM	Biopsy findings EM	Transplant/Recurrence	Hearing loss/Ocular disease
		1002	M	NA	NA	NA	?<44	U	GBM wrinkling FP effacement	N	N
		2000	M	NA	NA	NA	?<60	U		N	N
DUK6669	Cau	1	F	2	Y	Neg	U	U	U	N	N/N
		100	F	5	Y	Neg	U	U	U	N	N/N
		101	M	2	Y	Neg	U	U	U	N	N/N
		1000	M	U	Y	3+	U	U	U	N	N/N
		2000	M	20	Y	NA	U	FSGS	GBM Thickened GBM wrinkling FP effacement	Y/N	N/N

M, male; F female; Y , yes; N, No; U, unknown or not applicable; NA, data not available or not applicable AA, African American; Cau, Caucasian LM, Light Microscopy; EM, Electron Microscopy; GBM, Glomerular Basement Membrane; FP, Foot Process.

History of hearing loss/ocular disease established after identification of COL4A3 variants.

Table 2

COL4A3 or *COL4A4* variants found in the seven families.

Family	Gene	Variant	Genotype	Conserved Y/N	Polyphen Score [a]	SIFT Score [b]	Mutation Taster [c]	Novel Yes/rsID	MAF in EVS
DUK6696	COL4A3	del393G_E131fsX151	Compound	NA	NA	NA	NA	Yes	0
		2806C>T_Q936X	heterozygous					Yes	0
DUK6531	COL4A3	c4421C>T L1474P	Heterozygous	Y	1.0	0	98	rs200302125	0.003
DUK6534	COL4A3	c443G>T G148V	Heterozygous	Y	1.0	0	109	Yes	0
DUK6585	COL4A3	c4981C>T R1661C	Heterozygous	Y	1.0	0	180	rs201697532	0.0005
DUK6630	COL4A3	c2083G>A G695R	Heterozygous	Y	1.0	0	125	rs200287952	0.00017
DUK6527	COL4A4	c410G>A G137D	Heterozygous	Y	1.0	0	94	rs377511303	0.00008
DUK6669	COL4A4	C2906C>G S969X	Heterozygous	NA	NA	NA	NA	rs35138315	0.00008

NA, Not Applicable

MAF; Minor Allele Frequency; EVS, Exome Variant Server (NHLBI)

[a] Score range 0 – 1.0 with 1.0 most damaging

[b] Score range 0 – 1.0 with 0 most damaging

[c] Score range 0 – 215 with higher scores signifying more damaging variants (Grantham Index)