Article

**COL4A3 mutations cause focal segmental glomerulosclerosis**

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Focal segmental glomerulosclerosis (FSGS) is a histologically identifiable glomerular injury often leading to proteinuria and renal failure. To identify its causal genes, whole-exome sequencing and Sanger sequencing were performed on a large Chinese cohort that comprised 40 FSGS families, 50 sporadic FSGS patients, 9 independent autosomal recessive Alport’s syndrome (ARAS) patients, and 190 ethnically matched healthy controls. Patients with extrarenal manifestations, indicating systemic diseases or other known hereditary renal diseases, were excluded. Heterozygous COL4A3 mutations were identified in five (12.5%) FSGS families and one (2%) sporadic FSGS patient. All identified mutations disrupted highly conserved protein sequences and none of them was found in either public databases or the 190 healthy controls. Of the FSGS patients with heterozygous COL4A3 mutations, segmental thinning of the glomerular base membrane (GBM) was only detected in the patient with electronic microscopy examination results available. Five ARAS patients (55.6%) had homozygous or compound-heterozygous mutations in COL4A3 or COL4A4. Serious changes in the GBM, hearing loss, and ocular abnormalities were found in 100%, 80%, and 40% of the ARAS patients, respectively. Overall, a new subgroup of FSGS patients resulting from heterozygous COL4A3 mutations was identified. The mutations are relatively frequent in families diagnosed with inherited forms of FSGS. Thus, we suggest screening for COL4A3 mutations in familial FSGS patients.

**Keywords:** FSGS, mutation, COL4A3, COL4A4

**Introduction**

Focal segmental glomerulosclerosis (FSGS) is histologically characterized by focal and segmental glomerular sclerosis and foot-process effacement and its clinical manifestations include proteinuria and progressive renal failure. According to the available epidemiological data, FSGS accounts for nearly 20% of the nephrotic syndrome in children and adults. Among patients undergoing renal biopsies, it accounts for 24.3% and 3.3%–16% of primary glomerulonephritis (GN) in the USA and China, respectively (Xie and Chen, 2013).

The etiology of FSGS is largely unknown. However, changes in the filtration barrier, consisting of glomerular endothelial cells, basement membrane and visceral epithelial cells (podocytes), caused by either genetic or acquired factors are responsible for the severe proteinuria often observed in FSGS. FSGS can be a secondary outcome of systemic diseases, such as obesity, hypertension, and viral infections including HIV and parvovirus B19 (D’Agati et al., 2004), or hereditary renal diseases, such as Alport’s syndrome (AS) and Fabry disease (FD). Extrarenal manifestations are common in these patients. Familial segregation of FSGS indicates genetic involvement in its pathogenesis. Several monogenic FSGS subtypes have been reported by genetic studies primarily focusing on familial FSGS. Mutations in the *NPHS1* gene that codes nephrin, an essential component of the slit diaphragm, were identified in 1998 as the cause for Finnish congenital nephrosis (Kestilä et al., 1998). Since then, a series of causal genes of FSGS have been identified, including *NPHS2* (Karle et al., 2002; Caridi et al., 2003), *PLCE1*, *WT1* (Kozziell et al., 1999), *LAMB2* (Hasselbacher et al., 2006), *ACTN4* (Kaplan et al., 2000; Dai...
Figure 1 FSGS families with identified COL4A3 mutations. A green square indicates normal urine and serum creatinine levels; red in the upper left quarter indicates hematuria; yellow in the upper right quarter indicates proteinuria; blue in the bottom left quarter indicates renal function insufficiency; and blue in the bottom half indicates ESRD. (A) FS1 was defined by three brothers with biopsy-proven FSGS (FS1-201, 203, and 205). All three brothers had mild to moderate renal function impairment (Scr 117–167 μmol/L). The youngest brother (FS1-205) had NS range proteinuria.
et al., 2010), TRPC6 (Reiser et al., 2005; Winn et al., 2005; Zhu et al., 2009), CD2AP (Kim et al., 2003; Gigante et al., 2009), and INF2 (Brown et al., 2010). FSGS families possessing mutations in these genes consistently display an autosomal dominant (AD) or autosomal recessive (AR) inheritance pattern. However, these only account for a small portion of the familial FSGS patients (Boyer et al., 2011; Zhang et al., 2013). Moreover, a much lower mutation rate of these genes was reported in an Asian population (Zhang et al., 2013), suggesting that additional genes may be involved in FSGS pathogenesis. In this study, a large Chinese cohort was recruited and subjected to whole-exome sequencing followed by Sanger sequencing for the discovery of novel causal genes related to FSGS.

**Results**

**Clinical characteristics**

Forty independent FSGS families, 50 unrelated sporadic idiopathic FSGS patients, 9 clinically diagnosed ARAS families (21 individuals), and 190 unrelated ethnically matched healthy control individuals were enrolled for this study. Among all 40 FSGS families, three large families were selected for whole-exome sequencing. The rest 37 FSGS families and 50 sporadic FSGS patients were used for validations. The male to female ratio was 22:18 for index individuals in the familial FSGS patients and 30:20 in the sporadic FSGS patients. Age at renal biopsy (35.6 ± 15.2 vs. 39.7 ± 16.8 years) and disease onset (31.4 ± 14.6 vs. 35.6 ± 17.7 years) were similar between the familial and sporadic cases. No difference was observed in serum creatinine (log-transformed creatinine, 4.6 ± 0.8 vs. 4.8 ± 0.7 μmol/L), the frequency of ESRD (7.9% vs. 6.8%), or proteinuria (log-transformed urine protein, 0.22 ± 1.42 vs. 0.39 ± 1.33 g/24 h) between the familial and sporadic FSGS patients at the time of renal biopsy (Supplementary Table S2). The clinical and histological characteristics of the FSGS patients caused by COL4A3 mutations are summarized in Figure 1.

Nine ARAS patients (7 males and 2 females) from 9 families (21 members) were enrolled in this study. Of the nine patients, eight had microscopic hematuria and one had macroscopic hematuria. Proteinuria (0.39–3.97 g/24 h) was present in all the patients. Two patients had nephrotic syndrome. Six patients developed sensorineural deafness, and one patient had a lens abnormality. The clinical and histological characteristics of the ARAS patients caused by COL4A3 or COL4A4 mutations are summarized in Figure 2.

**Whole-exome sequencing**

Fifteen individuals were selected for exome sequencing, including nine patients and six unaffected individuals from three relatively large FSGS families. For each participant, ~44017835 bases were created and covered on the target. The sequence data were generated with a 177 × average coverage for each subject. An average coverage of the target region was 99.46%, and 98.97% of the target region had at least 4 × coverage. For each participant, ~21134 single-nucleotide variants were found, of which ~9876 were missense mutations and ~122 were nonsense (premature termination) mutations (Supplementary Table S1).

**Variants of COL4A3 in FSGS families**

The genomic DNA from three brothers (FS1-203, 205, and 207) and their parents (FS1-101 and 102) in family FS1, DNA samples from four sisters (FS2-201, 202, 204, and 205) and husband (FS2-206) and daughter (FS2-305) of the index (FS2-205) in FS2, and DNA samples from five relatives (pedigree tree not provided) in FS7 were subjected to exome sequencing. Two heterozygous missense COL4A3 mutations were found in families FS1 and FS2. Next, Sequenom MassArray was used to validate the variants in all family members. We verified perfect disease co-segregation with c.8847G>A (p.Cys1616Tyr) missense variant in FS1 and c.2401G>A (p.Gly801Arg) in FS2 (Figure 1).

(5.5 g/24 h). Non-NS range proteinuria (0.5–2.1 g/24 h) was detected in the other two brothers and one of their descendants (FS1-302), all in their twenties to forties. (B) FS2 was identified in two sisters with biopsy-proven FSGS (FS2-204 and 205). They, together with their elder sisters (FS2-201 and 203) were clinically characterized by non-NS range proteinuria (0.5–2.1 g/24 h). Two of their descendants (FS2-304 and 305) had microalbuminuria and microscopy hematuria. The eldest sister (FS2-201) had ESRD and started hemodialysis in her sixties. The youngest sister (FS2-205) had progressive renal dysfunction (Scr 134–250 μmol/L) when the renal biopsy was performed. (C) FS3 was a small family with two affected individuals. Both the index patient (FS3-201) and his mother (FS3-102) had experienced microalbuminuria in their second to fifth decade, and renal function was normal at the time of the family survey. (D) FS4 was defined by a female FSGS patient (FS4-301) and her mother (FS4-203) with moderate to severe proteinuria (2.7–3.5 g/24 h). Her grandmother (FS4-102) had edema and hematuria in her thirties. Severe proteinuria was found, and she died shortly after the diagnosis. The son of the index individual was a 15-year-old boy (FS4-401) and was found to have microalbuminuria at the time of the family survey. (E) FS5 was defined by a young woman (FS5-302) with heavy proteinuria (10.2 g/24 h). She had progressive proteinuria at the age of 19, and a renal biopsy was performed at age 35. Her mother (FS5-204) also had proteinuria (0.37 g/24 h) in her fifties. They both had normal renal function and blood pressure. (F) Summary of the clinical characteristics of the five FSGS families and the one sporadic FSGS patient with COL4A3 mutations. (G and I) Renal biopsy light micrographs from individuals with FSGS showing typical focal segmental sclerosis (arrow). (H) Renal biopsy electron micrographs from individuals with FSGS demonstrating effacement of podocyte foot processes and segmental thinning of the GBM (arrow). FID, familial ID; IID, individual ID; AO, age at onset; Pro, proteinuria (g/L); ACR, urinary albumin/creatinine ratio (mg/μmol); Scr, serum creatinine (μmol/L); Bp, blood pressure (mmHg); RB, renal biopsy; Mut, mutation; PS, PolyPhen2 Score; Ska5, α5 type IV collagen of SKIN; GMAa5, α5 type IV collagen of the GBM; BDa5, α5 type IV collagen of Bowman’s capsule and distal tubular basement membrane; GBDA3, α3 type IV collagen of the GBM, Bowman’s capsule, and distal tubular basement membrane; EM, electronic micrograph of the GBM; ST, segmental thinning; DT, diffused thickening, thinning and lamellation; n.a., not available; Nor, normal.
Screening for COL4A3 and COL4A4 variants in additional FSGS cases

We identified four more novel variants of COL4A3 in three additional FSGS families and one sporadic case, respectively (Figure 1). The variants c.352G>A (p.Gly118Arg), c.2990G>A (p.Gly997Glu), and c.2210T>A (p.Leu737His) co-segregated with FSGS in FS3, FS4, and FS5, respectively. The other missense mutation (c.2827G>A), resulting in a p.Gly943Arg substitution, was found in a sporadic case (sFS46). Then the presence of these novel variants in 190 ethnically matched controls was assessed by sequencing the corresponding sites using Sanger sequencing or Sequenom Spectrum MassArray. None of these six variants was detected in the control individuals. Using an evolutionary conservation analysis, we found that all six variants were located in a highly conserved region of COL4A3 (Figure 3B). Additionally, no additional mutations were detected when sequencing the COL4A4 gene in these families (Figure 1).

Next, the clinical characteristics were compared between the index patients with and without mutations in COL4A3. No difference in the age, gender, or disease severity parameters was detected across all the FSGS families (Supplementary Table S2). Segmental thinning of the glomerular basement membrane (GBM) was detected in four FSGS families (FS1, FS2, FS3, and FS4), while no obvious GBM changes were found in FS5 or sFS46.

Additionally, there were no diffuse GBM changes, basket weaving, or irregularity of the GBM’s epithelial surface in any of the available biopsies.

Screening for COL4A3 and COL4A4 mutations in ARAS patients

Two ARAS patients had a homozygous mutation in COL4A3 (p.Gly619Arg in AP4) or COL4A4 (p.Tyr1533Stop in AP3). In both cases, the parents were asymptomatic carriers, and the AP3 family had a history of consanguinity. The following compound-heterozygous mutations were detected in families AP2 and AP5: p.Gln1323Stop plus p.Gly957Arg in COL4A4 in AP2 and p.Cys1513Stop plus chr:227942770delG in COL4A4 in AP5. A single-nucleotide deletion, chr:22817249delA, which resulted in a frame shift in COL4A3, was identified in a 6-month-old infant (AP1). None of these variants were found in the 190 ethnically matched controls.

Figure 2 Clinical and histological characteristics of the ARAS families. (A and C) Renal biopsy light micrographs from individuals with ARAS showing mild focal segmental mesangial cell proliferation (AP1) or focal segmental sclerosis lesions (AP4). (B and D) Renal biopsy electron micrographs from individuals with ARAS demonstrating diffuse thickening and lamellation of the GBM (arrow). (E) Summary of the clinical characteristics of the five ARAS patients with COL4A3/COL4A4 mutations. HT, hearing test; EE, eye examination; SH, sensorineural hearing loss; LA, lens abnormality; MD, macular degeneration; neg, no mutation was found.
The total \( \text{COL4A3} / \text{COL4A4} \) mutation rate in the ARAS families was estimated to be 55.6% (5/9), which is similar to previous reports (Storey et al., 2013).

The five ARAS patients with \( \text{COL4A3} / \text{COL4A4} \) mutations were four males and one female, with disease onset at ages from 6 months to 12 years. The EM findings revealed lamellation and diffuse thinning and thickening of the GBM. Immunostains for type IV collagen \( \alpha_1 \), \( \alpha_5 \) chains in the epithelial basement membranes (EBMs) were positive and continuous among the three patients with available data (AP1, AP2, and AP3). Staining for type IV collagen chains in kidney tissue by using monoclonal anti-\( \alpha_1 \), \( \alpha_3 \), \( \alpha_5 \)(IV) chain antibodies (Wieslab Co.) was available in four patients (AP1, AP2, AP3, and AP4). They all had negative staining for the \( \alpha_5 \)(IV) chain in the GBM, positive staining for \( \alpha_5 \)(IV) chain in Bowman’s capsule and distant tubules, and negative staining for \( \alpha_3 \)(IV) chain in the GBM, Bowman’s capsule, and distant tubules (Figure 2). Sensorineural hearing loss was found in four patients (AP1, AP3, AP4, and AP5), and eye abnormalities were found in two patients, including lens abnormality (AP2) and macular degeneration (AP5) (Figure 2).

**Discussion**

Several mutations in podocyte-related genes have been recognized as causes for FSGS in the last decade (Kaplan et al., 2000; Kim et al., 2003; Reiser et al., 2005; Winn et al., 2005; Gigante et al., 2009; Brown et al., 2010). However, the known mutations only account for a small portion of familial FSGS, indicating that additional genetic factors most likely exist. In our study, we performed exome sequencing and subsequent targeted sequencing of a cohort that consisted of 40 FSGS families and 50 independent FSGS patients with variable severity of adult-onset proteinuria and chronic renal failure. We identified six independent conservative missense mutations in \( \text{COL4A3} \) that are most likely responsible for the observed FSGS phenotype. The mutations altered highly conserved amino acid residues in the \( \alpha_3 \) chain of type IV collagen. Five of the six detected mutations segregated precisely with renal disease in five unrelated FSGS families. The sixth heterozygous mutation was found in a sporadic FSGS patient. Our data suggest that \( \text{COL4A3} \) mutations account for 12.5% of the autosomal dominant FSGS cases in China. A recent study by a group at Duke University was published (Malone et al., 2014), when this manuscript was in...
GBM thinning, in some FSGS patients with COL mutations would break the collagen backbone. Interestingly, identified in other four of the six mutations are glycine mutations, which were also present in the trimeric noncollagenous domain and the other five are located in the collagen domain. In aggregate, these findings are clearly relevant to both Caucasian and Chinese families with FSGS. We also found that, in rare cases, COL4A3 mutations may explain sporadic FSGS. These results highlight that the diagnosis of FSGS based on purely descriptive histopathologic criteria may have a highly heterogenous genetic etiology.

COL4A3 and COL4A4 are two genes located head to head on 2q36–37 and code for the α3 and α4 collagen chains of type IV collagen, which is a basement membrane-specific protein. Heterozygous carriers of either COL4A3 or COL4A4 mutations have been reported as the causes for thin basement membrane disease (TBMD) and autosomal dominant Alport’s syndrome (ADAS) (Pochet et al., 1989; Beirowski et al., 2006; Longo et al., 2006; Abrahamson et al., 2009). TBMD, defined by diffuse thinning of the GBM, as determined by electronic microscopy (EM), frequently causes persistent microscopic hematuria. Proteinuria or chronic renal failure is unusual, and progression to ESRD is considered rare. Heterozygous mutations in COL4A3/4A4 were reported as causes for TBMD in several large Cypriot pedigrees (Yoskarides et al., 2007; Pierides et al., 2009).

In this study, we detected TBMD-like changes, such as segmental GBM thinning, in some FSGS patients with COL4A3 mutations. However, typical changes, such as diffuse GBM thinning, were not found. Prior studies have suggested that heterozygous COL4A3 or COL4A4 mutations are not benign and may lead to ADAS (Jefferson et al., 1997; van der Loop et al., 2000; Chatterjee et al., 2013). Other studies have argued that ADAS most likely represents TBMD in combination with another renal disease, such as IGN (Cosio et al., 1994). The diagnosis of ADAS is reserved for individuals with a lamellated GBM in combination with extrarenal manifestations, such as high-tone sensorineural deafness or ocular defects (Lemmink et al., 1997; Kashtan, 1999; Savage et al., 2013). Consequently, the ADAS diagnosis is extremely rare (Pochet et al., 1989; Jefferson et al., 1997; van der Loop et al., 2000; Ciccarese et al., 2001; Longo et al., 2006). Our study expands the phenotypic spectrum of renal diseases in COL4A3 mutation carriers and demonstrates that these patients may be frequently misdiagnosed as primary FSGS. Notably, although we found that heterozygous COL4A3 mutations could lead to FSGS in a sporadic case, we were unable to exclude the existence of asymptomatic proteinuria or kidney diseases in his family members.

The mechanism by which mutations in COL4A3 lead to FSGS remains unknown. Among the six mutations we identified in this study, one (Cys1616Tyr in FS1) is in the NC1 (trimeric noncollagenous) domain and the other five are located in the collagen domain. Four of the six mutations are glycine mutations, which were also identified in other COL4A3-related diseases. Glycine forms alpha helix structures in proteins, and disruption of the alpha helix would break the collagen backbone. Interestingly, COL4A3 mice develop both TBMN and FSGS-like lesions (Beirowski et al., 2006). Our study found that heterozygous mutations of COL4A3/COL4A4 cause FSGS without diffuse GBM changes or extrarenal involvement. It is known that collagen IV α3, α4, and α5 chains assemble to form helical heterotrimeric in the endoplasmic reticulum (ER) of podocytes but not in endothelial or other adjacent cells (Abrahamson et al., 2009). Studies have found that mutations of COL4A3/COL4A4 in the NC1 domain disrupt heterotrimer formation in the podocyte and subsequent secretion into the GBM. The putative intracellular effects due to the accumulation of misfolded proteins inside the podocyte were also evidenced by a recent study (Pieri et al., 2014), which showed that mutant COL4A3 chains retained in the ER were associated with activation of the unfolded protein response (UPR) pathway (Yoshida, 2007), leading to cytotoxicity and apoptosis (Rajpar et al., 2009). Additionally, collagen defects may cause podocyte detachment, leading to FSGS. However, the exact mechanisms underlying the disease require further investigation.

In summary, our findings suggest that a large fraction of familial FSGS can be explained by heterozygous COL4A3 mutations. Therefore, we recommend clinical COL4A3 mutation screening in familial FSGS patients. Considering the large size of COL4A3 gene, next-generation sequencing (NGS) approaches may be preferred over standard Sanger sequencing.

Materials and methods

Patients

The diagnosis of FSGS is defined by the presence of typical segmental sclerosis in some glomeruli in the renal biopsy specimen. Familial FSGS is defined as one of the following conditions: (i) two or more blood relatives diagnosed with FSGS by renal biopsy; (ii) at least one family member with a biopsy diagnosis and one or more blood relatives with proteinuria, microalbuminuria, progressive renal dysfunction, or ESRD of unknown cause. The exclusion criteria include: (i) evidence of systemic diseases, such as obesity-related glomerulopathy, hypertensive nephropathy, and HIV infection; (ii) extrarenal manifestations, including hearing loss and eye problems suggestive of a hereditary kidney disease, such as Alport Syndrome (AS), thin basement membrane disease (TBMD), and Fabry disease; (iii) known mutations in the ACTN4, TRPC6, and INF2 genes.

The diagnosis of ARAS is determined according to the following most recent guidelines (Savage et al., 2013): (i) presence of hematuria or proteinuria or both, progressive renal failure with or without sensorineural hearing loss or ocular lesions; (ii) kidney injury found in a single generation or a consanguineous family; (iii) males and females in the family are equally affected; (iv) characteristic ultrastructural changes in the GBM; (v) characteristic immunofluorescence staining of type IV collagen chains in the renal basement membranes, including negative staining for α5(IV) chain in the GBM, positive staining for α5(IV) chain in Bowman’s capsule and distal tubules, and negative staining for α3(IV) chain in all GBM, Bowman’s capsule, and distal tubules.

Demographic, clinical, and pathological data were recorded, including age, disease onset age, gender, body mass index (BMI), serum creatinine, uric acid, hemoglobin, blood pressure,
24-h protein excretion, enzymatic activity of lysosomal enzyme α-galactosidase A, visual and hearing test, and staining of kidney and skin tissues for α1, α3, and α5 type IV collagen. Renal biopsy samples were used for light, immunofluorescence, and electronic microscopy evaluations. Blood samples were collected from all available family members for genomic DNA extraction. All the subjects signed an informed consent, and the study was approved by the Human Research Ethics Committee of Shanghai Ruijin Hospital, Shanghai Jiao Tong University, School of Medicine.

**Exome sequencing**

Exome sequencing was performed by BGI (Shenzhen, China). Six micrograms of sample DNA were prepared. First, the qualified DNA samples were randomly fragmented to generate 200–300 bp DNA fragments. The extracted DNA was amplified in a ligation-mediated polymerase chain reaction (LM-PCR). The NimbleGen human exome array (SeqCap EZ Human Exome Library v2.0) was used to capture the exons of the human genome. High-throughput sequencing was performed on a HiSeq2000 platform (Illumina), and the sequence of each library was generated as 90 bp paired-end reads. The raw image files were processed by Illumina base call Software 1.7 (HCS1.5.15.1, RTA1.13.48, OLB 1.9.4). The obtained sequences were aligned to the reference genome [human genome build37 (hg19)] using Burrows-Wheeler Aligner (BWA) (Version: 0.5.9-r16). Single-nucleotide polymorphisms (SNPs) were detected by SOAPsnp (Version: 1.05) and small insertions/deletions (indels) were detected by SAMtools (Version: 0.1.18). Called SNP variants and indels were annotated and classified using ANNOVAR [Last Update: 2011-10-02]. We focused on the variants that were shared by all the affected subjects and absent from the unaffected relatives. Variants in dbSNP132, dbSNP132, dbSNP137, and the 1000 Genomes project with a frequency higher than 0.001 in the Asian population or in our reference sequencing dataset from 400 normal controls were excluded. Candidate variants were validated by Sequenom Spectrom MassArray. A segregation analysis was performed in each family.

**Sequenom MassArray**

The Sequenom MassArray platform is a powerful tool for SNP genotyping, quantitative gene expression, and methylation studies. MassArray SNP Genotyping combines the benefits of simple and accurate primer extension chemistry with state-of-the-art MALDI-TOF mass spectrometry and is a quick and cost-effective way to characterize genotypes with a high level of reproducibility. SNP Genotyping was conducted using the Sequenom MassArray iPLEX Platform following the manufacturer’s protocol. First, PCR primers and extension probes were designed using the Sequenom online primer design system. Then, PCR amplification and single-base extension were conducted. Finally, the purified extension products were dispensed onto a Spectro-CHEP bioarray, and a MALDI-TOF mass spectrometer distinguished the different bases according to their mass. The data were analyzed using the MassArray Workstation software (Typer4.0). In this study, both Sequenom MassArray and Sanger sequencing were used to validate the exome sequencing data.

**Amplification and sequencing of COL4A3 and COL4A4**

Multiplex PCR sequencing of the COL4A3 and COL4A4 coding exons was conducted in additional 37 FSGS families and 50 sporadic FSGS cases. PCR and sequencing primers are listed in Supplementary Tables S3 and S4. We sequenced these specimens using Ion AmpliSeq™ at WuXi AppTec (Shanghai, China). Ion AmpliSeq™ Technology was used to conduct ultrahigh-multiplex PCR with a low DNA input requirement. The ultrahigh-multiplex PCR amplified the target sequence, and the Ion PGMTM Sequencer generated the whole exon sequences of the COL4A3 and COL4A4 genes. Next-generation sequencing technology is effective and convenient to scan a target gene for genetic study. The rare variants that we identified in the COL4A3 and COL4A4 genes were also validated by Sanger sequencing.

**Statistical analysis**

The distributions of the quantitative variables were summarized as the means and standard deviations (or the medians and ranges for non-normally distributed variables). Statistical testing of the continuous variables was performed using Student’s t-test (or the Mann–Whitney U test if appropriate). All categorical variables were expressed as frequencies or percentages (%), and a comparison of the proportions was performed using a standard X² test.

**Supplementary material**

Supplementary material is available at Journal of Molecular Cell Biology online.

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**Conflict of interest:** none declared.

**References**


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