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Original Article

Novel deletion mutation in a Chinese family with X-linked alport syndrome

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Abstract: Backgrounds and Objectives: alport syndrome (AS) is a progressive hereditary condition that is characterized by haematuria, proteinuria, progressive renal impairment, and end stage kidney disease (ESRD). Approximately 85% of AS patients have X-linked mutations in the COL4A5 gene that encodes type IV collagen. The aim of our study was to identify the gene responsible for glomerulopathy in a 3-generation Chinese pedigree with familial haematuria. Methods: We examined five members of a Chinese family clinically suspected of X-linked AS caused by COL4A5 gene mutations. All 51 exons of the COL4A5 gene were screened by direct DNA sequencing. Results: We identified the novel deletion mutation c. 3990_4016delCCC...TCC in COL4A5 in three affected individuals with haematuria, but the mutation was absent in the other 2 healthy family members and 100 unrelated healthy controls. Conclusions: Our result demonstrates that the mutation is pathogenic and novel and has meaningful implications for the diagnosis and genetic counselling of cases with AS. The results in the study broaden the genotypic spectrum of known mutations for AS.

Keywords: Alport syndrome, haematuria, COL4A5, child

Introduction

Familial glomerular haematuria is typically caused by thin basement membrane nephropathy (TBMN). Considerably less often, this condition is due to alport syndrome (AS). The recognition of AS is relatively more important because it results in a higher rate of progression to end-stage renal disease (ESRD) [1-3].

The incidence of AS is approximately 1-10: 50,000 [4], and it can lead to ESRD, with an age-associated risk of onset: 50% by age 25, 90% by age 40, and nearly 100% by age 60 [5]. There is no radical cure for AS patients [6], and there is evidence that treatment with ACE inhibitors even before the onset of microalbuminuria slows down the rate of progression to ESRD [7]. Therefore, a timely diagnosis of AS has a high impact on the prognosis.

AS is a monogenic disease of the basement membrane (BM) and is caused by defects in type IV collagen, a major structural component of BM that is necessary for BM maintenance [8]. X-linked AS (XLAS) accounts for 85% of AS cases and is attributed to mutations in one of the three genes encoding type IV collagen α-chain isoforms (α3, α4, and α5) [9].

There are genotype-phenotype correlations in XLAS [3, 9]. Patients with various variants in the COL4A5 gene may present with a wide spectrum of phenotypes, ranging from benign familial haematuria (BFH) or TBMN to ESRD. Not all AS individuals present with the typical clinical features including haematuria and/or proteinuria, accompanied by hearing impairment and ocular lesions. Findings from light microscopy are also often non-specific. In addition, electron microscopy (EM) of the glomeruli is not always available. Thus, diagnosing AS histologically can be challenging.

Skin biopsy, which is much less invasive than kidney biopsy, can be highly informative for most XLAS cases [10, 11] because the collagen α5 (IV) chain is normally present in the skin basement membrane. However, a segmental pattern of expression or normal expression by

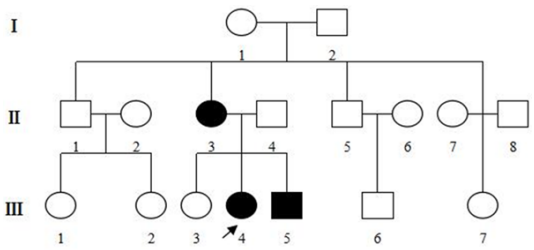

Figure 1. Pedigree of the presenting family. Multiple family members have mutations in the COL4A5 genes (black, c.3990_4016delCCC...TCC) and show hematuria. Arrow: Proband. Affected individuals with hematuria are shown as blackened squares (males) and circles (females). Normal individuals are shown as empty squares (males) and circles (females). Crossed squares or circles denote deceased individuals.

immunofluorescence studies can be observed in female carriers [12, 13]. Genetic screening is generally replacing more invasive investigations such as kidney biopsy and skin biopsy. It has emerged as the gold standard in making a definitive diagnosis at a molecular level in approximately 95% of patients who have AS [14-16], in favour of timely pre-emptive therapeutic intervention, particularly for cases with isolated haematuria without extrarenal manifestation.

In this study, we analysed COL4A5 mutations using direct DNA screening and disease features in a Chinese family with haematuria, with or without proteinuria, who were suspected of having XLAS. Neither sensorineural hearing loss nor ocular lesions were present in any member of this family. Moreover, we identified a novel pathogenic deletion mutation c.3990_4016delCCC...TCC in the COL4A5 gene in the three affected individuals. To the best of our knowledge, this report is the first to describe the novel deletion mutation in the COL4A5 gene.

Materials and methods

Subjects

A 17-member Chinese family including 3 generations of Hunan ethnicity was enrolled in this study (**Figure 1**). Clinically asymptomatic glomerulopathy in members of this family was ruled out if the urinalysis identified no more than trace amounts of blood or protein [17]. Peripheral blood samples were collected from 5 members in the family including 3 patients and 100 unrelated, healthy controls of the same ethnicity.

Clinical data collection

The 5 members of the patients' family were assessed for haematuria, proteinuria, and renal function, which is the cornerstone of the diagnosis of renal pathology associated with AS [18]. In addition, all 5 members underwent clinical examination by an ophthalmologist and otolaryngologist. Ocular examination included testing for visual acuity, anterior lenticonus, cataracts, and optic disc or retinal abnormalities. Sensorineural hearing loss was assessed using standard audiometry tests. Volunteers with vision and/or hearing abnormalities, hypertension, or blood concentrations of creatinine or urea outside of normal levels were excluded from the study.

Five micrometre-thick sections from frozen skin tissues were used for the immunofluorescent staining of type IV collagen. Double fluorochrome immunofluorescence staining for the alpha-5 and alpha-1 chains of type IV collagen was performed using fluorochrome-conjugated monoclonal antibodies (anti-type IV collagen α5 chain, human (Mono), cosmo bio, Japan and wieslab™ alport syndrome kit, wieslab, sweden) for AS. The skin sections were reacted with fluorescein isothiocyanate conjugated-anti-type IV collagen alpha-5 chain and texas-red-anti-type IV collagen alpha-chain and then observed under immunofluorescence microscopy.

Kidney biopsy

A kidney biopsy was performed for the proband. A formalin-fixed paraffin-embedded biopsy was performed, 3 mm sections were cut and stained by H&E, and the periodic acid-Schiff reaction and Masson's trichrome methods were used. Pieces of renal cortex were processed for electron microscopy, and semi-thin sections were stained with toluidine blue and examined by light microscopy to select the appropriate glomeruli. Next, 50 nm sections were cut, stained with uranyl acetate and lead citrate and examined with a Zeiss Leo 906 E electron microscope equipped with a Megaview III digital camera (Oberkochen, Germany). For routine immunofluorescence evaluation of the renal

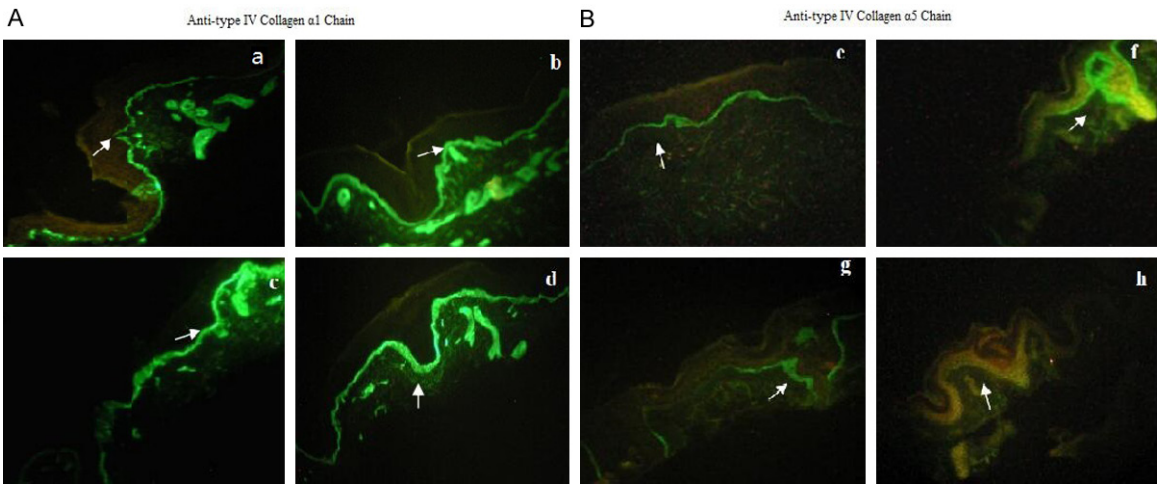

Figure 2. Distribution of the alpha-1 and alpha-5 chains of type IV collagen in the epidermal basement membrane. Localization of the alpha-1 and alpha-5 chains was expressed as green fluorescence (indirect label immunofluorescent technique, original magnification × 200): A and B. An alpha-1 chain was stained in the normal linear pattern in the siblings III 4 and 5 and their mother II-3 (b, c, d, arrows); normal mouse (a, arrows). An alpha-5 chain was distributed diffusely but weakly in the membrane of the mother II-3 (f, arrowheads) and the proband (g, arrows). The absence of a type IV collagen a5 chain by immunohistochemistry in the proband's younger brother (h, arrows). Normal mouse control (e, arrows).

biopsy, one fragment was embedded in optimal cutting temperature medium and snap frozen in liquid nitrogen. Then, 5-mm thick frozen sections were cut and sequentially fixed in acetone, after which they were washed with phosphate buffered saline (PBS), stained with fluorescein isothiocyanate conjugated antisera (Kallestad, Austin, TX, USA) against human immunoglobulin G (IgG), IgM, IgA, C3, C1q and κ and λ light chains diluted 1:20, washed in PBS and mounted with buffered glycerol. Furthermore, the expressions of the α1 chain, α3 chain and α5 chain collagen type IV were analysed using an Alport Syndrome Staining Kit (ALPOC diagnostics TM). Negative (without primary antibody) and positive (normal kidney) controls were also analysed.

DNA sequencing

DNA was isolated from the peripheral leukocytes of the five family members using the DNA purification kit (TIANgene, Beijing, China) according to the manufacturer's protocol. All 51 exons and intron-exon junctions in COL4A5 were amplified by PCR. The primers were designed by the primer 5 programme (Supplementary Table 1). The mutational analysis was performed by direct DNA sequencing. The PCR products were run on 1.2% agarose gel and sequenced with both forward and reverse primers. DNA sequencing was performed using the BigDye Terminator Cycle Sequencing v3.1 kit (ABI, Foster City, CA). Sequences were detected using the 3730 XL Genetic Analyzer (Applied Biosystems, Life Technologies, CA) to screen for pathogenic mutations in the family.

Evaluation of pathogenicity

The pathogenic potential of all identified mutations and sequence variants was analysed by DNAStar v5.01. An analysis of abnormal amino acid sequence encoded by detected variants was performed using DNAMAN v6.0.3. All mutations identified in this study have been checked against HGMD (http://www.hgmd.cf.ac.uk/ac/) and LOVD (http://www.lovd.nl/3.0/home), Moreover, all novel mutations identified in this study were excluded in 100 ethnically matched control chromosomes.

This study was approved by the Human Research Ethics Committee of the Second Xiangya Hospital, Central South University, and all participants signed informed consent.

Results

The affected familial pedigree consisted of 17 individuals across 3 generations. The proband

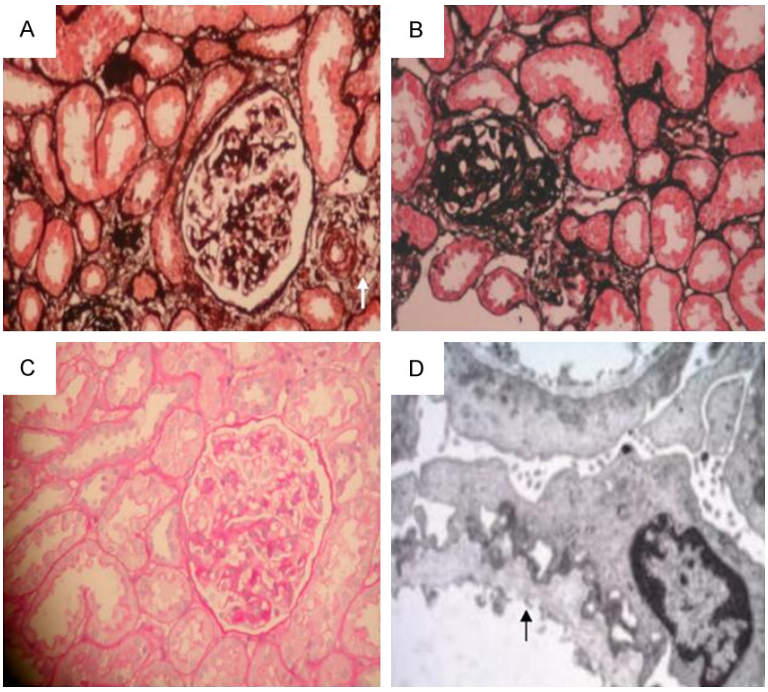

Figure 3. Light and electron microscopy pictures of the renal biopsy taken from the proband. A. Light microscopy, original magnification × 200. PASM staining. Shown is a glomerulus with segmental sclerosis, consistent with an FSGS lesion. Small arteries show loss of smooth muscle fibres (arrow) and fibrosis in the interstitium. B. Light microscopy, original magnification × 200, PASM staining. Shown is a globally sclerotic glomeruli and fibrosis in the interstitium. C. Light microscopy, original magnification × 200, periodic acid-Schiff staining. Shown is a glomerulus with segmental sclerosis and moderate segmental mesangial expansion, consistent with an FSGS lesion. D. Electron microscopy, original magnification × 230,000. Shown is the lamellated GBM (l, black arrow).

is a 4-year-old girl with haematuria and proteinuria and a familial history of haematuria.

She was found to have haematuria by neighbouring doctors. She was referred to our hospital for further evaluation of persistent haematuria (2 months). The physical examination revealed no abnormalities, and she did not suffer from hypertension (98/66 mmHg), eye involvement, or sensorineural deafness. Her urine analysis showed mild to moderate proteinuria (urinary protein ++, urinary microalbuminuria > 360 mg/L) and microscopic haematuria (erythrocytes 89,000/high power field, 72% of glomerular origin). A clinical laboratory analysis revealed the following: total protein 44.5 g/l, uric acid 245.9 μmol/L, TG 3.17 mmol/L, blood urea nitrogen 3.84 mmol/L, and creatinine71.2 μmol/L; all of these results were normal. The complete blood counts were normal (leukocytes, 4-5 × 10^9/L; erythrocytes, 11-12 × 10^9/L), and anti-neutrophilic cytoplasmic antibodies were negative. The renal ultrasonography was also normal.

She was full-term at birth as the second child of unrelated Chinese parents. The family history was remarkable (**Figure 1**). Her mother and younger brother also have haematuria. Her younger brother had first presented as a 2-year-old with episodes of macroscopic haematuria without other symptoms. Laboratory tests revealed the following characteristics: erythrocytes 290,000/high power field, 70% of glomerular origin, urinary protein-, urinary microalbuminuria > 255.44 mg/L. Hepatic and renal functions were normal, and plasma lipids were normal. Her mother, II-3, was 40 years old at the time of the study. Her urinalyses and blood chemistry disclosed microscopic haematuria (10,000/high power field, 90% of glomerular origin) and proteinuria (urinary protein+, urinary microalbuminuria 313.46 mg/L); none of these patients had ESRD, sensorineural hearing loss, or eye complications such as lenticonus.

The skin tissues obtained from the proband (III:4) and her mother (II-3) revealed a weak distribution of the alpha-5 chain of type IV collagen in the epidermal basement membranes (**Figure 2**), whereas the skin tissues obtained from her younger brother (III:5) were negative for the alpha-5 chain of type IV collagen. The distribution of the alpha-1 chain of type IV collagen was normal in all 3 affected family individuals.

The proband's light microscopy examination demonstrated global and segmental sclerosis and mesangial expansion (**Figure 3**); moreover, PASM staining showed segmental sclerosis in 4 out of 16 glomeruli (**Figure 3A**), and globally sclerotic glomeruli were present in 2 of 16 glomeruli (**Figure 3B**). Moderate segmental

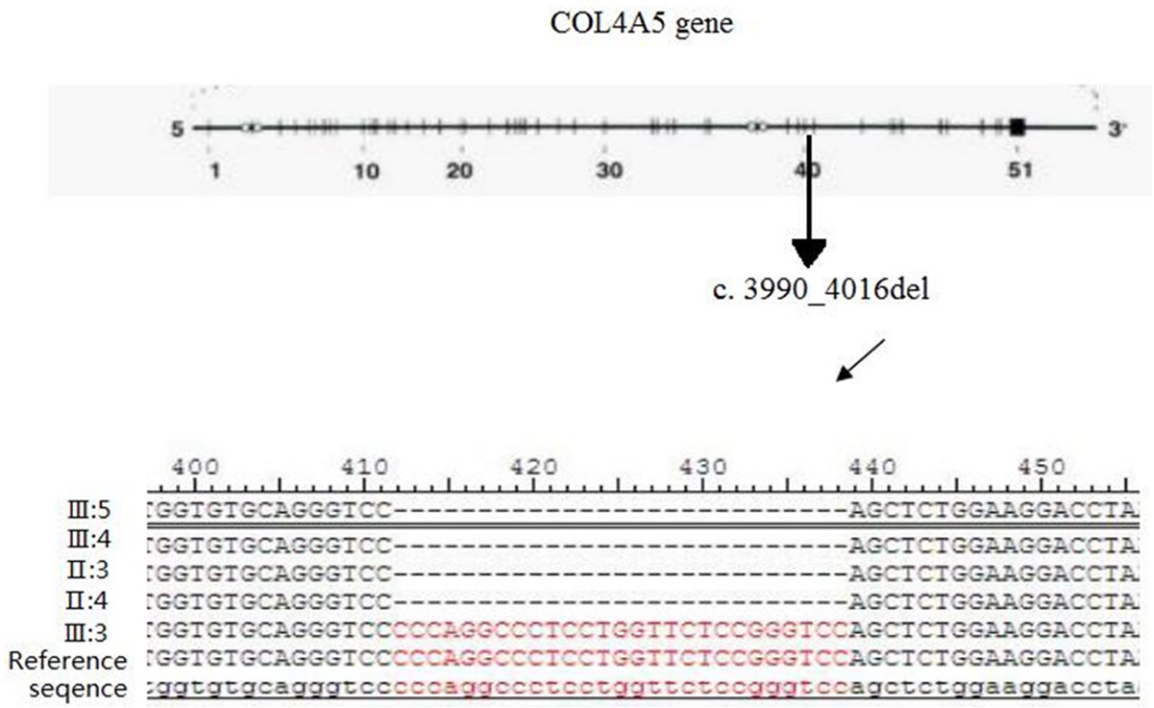

Figure 4. Sequencing analysis of mutation c. 3990_4016del in the COL4A5 gene. A Heterozygous c. 3990_4016del mutation was identified in the three affected individuals (III:5, III:4, II:3).

mesangial expansion was also present (**Figure 3C**). There was mild patchy interstitial fibrosis and partial renal tubular atrophy; a few tubular epithelial cells displayed deformation necrosis, and individual tubular cavities had red blood casts. However, lipid-laden foam cells, a marker of AS, were not found. Electron microscopy revealed a glomerular basement membrane (GBM) with a lamellate appearance, subepithelial frilling, small intramembranous deposits and uneven thickness (**Figure 3D**), and there was an extensive effacement of podocyte processes (not shown). Immunofluorescence studies for the IgG, IgA, IgM, C3 and C1q and κ and λ light chains revealed no significant deposition of these reactants in the glomeruli, the tubular basement membranes, or the interstitium. The results of the sequence analysis of the COL4A5 gene disclosed a deletion mutation for c.3990_4016delCCC...TCC in exon 41 in the proband, her younger brother, and their mother (**Figure 4**); this deletion was absent in the unaffected members (her father or her elder sister) and 100 ethnically matched normal controls by direct sequencing. This mutation has not been described before by HGMD and LOVD. Moreover, the large deletion we identified is a highly conserved amino acid residue amongst different species from humans to zebrafish (human, mouse, rat, xenopus, and zebrafish) (**Figure 5**).

The mutation we identified causes a 9 amino acid deletion of Proline to Proline (Proline-Glycine-Proline-Proline-Glycine-Serine-Proline-Glycine-Proline) at positions 1237-1245 of the protein codified by the COL4A5 gene (**Figure 6**), which results in secondary structure changes at positions 1201-1260 of the protein (**Figure 7**). Thus, this mutation was predicted to affect the protein features and be disease causing.

Discussion

In this study, we investigated a Chinese family of Hunan nationality with XLAS. Through direct DNA sequencing, we identified a novel mutation c. 3990_4016delCCC...TCC in the COL4A5 gene in three family members; the mutation was not present in healthy family members or the 100 healthy control individuals.

Haematuria, proteinuria, renal failure, high tone sensorineural hearing loss, characteristic ocular abnormalities (anterior lenticonus, a central fleck retinopathy or peripheral coalescing reti-

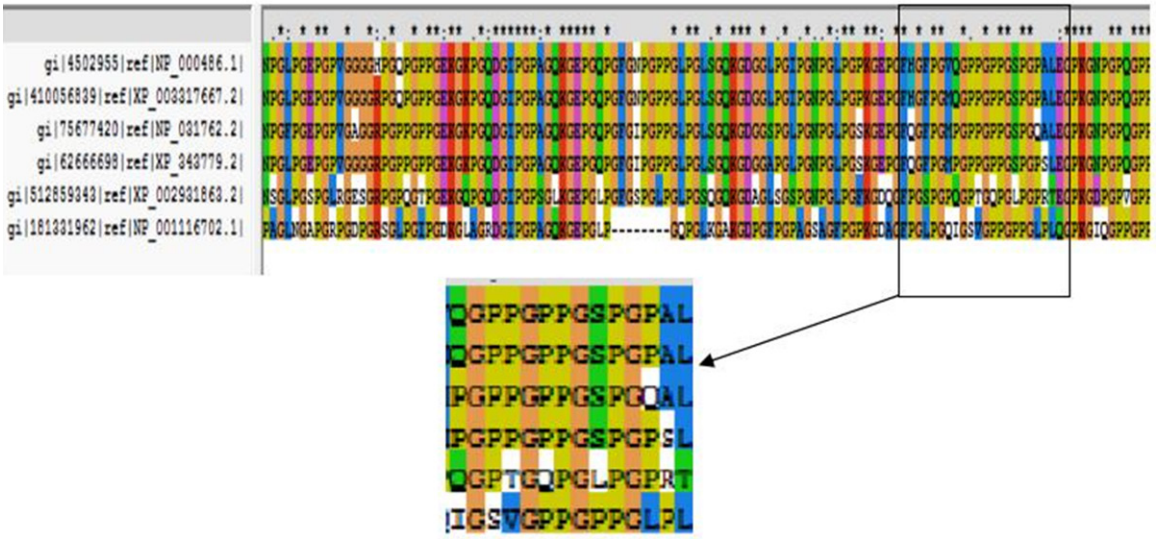

Figure 5. Conservation analysis of *COL4A5* p.Pro167 amino acidresidue. Conservation analysis of the *COL4A5* c. 3990_4016del amino acid residue in different species.

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ORIGIN
1       MKLRGVSLAA GLFLLALSLW GQPAEAAACY GCSPGSKCDC SGIKGEKGER GFPGLEGHPG
61      LPGFPGPEGP PGPRGQKGDD GIPGPPGPKG IRGPPGLPGF PGTPGLPGMP GHDGAPGPQG
121     IPGCNGTKGE RGFPGSPGFP GLQGPPGPPG IPGMKGEPGS IIMSSLPGPK GNPGYPGPPG
181     IQGLPGPTGI PGPIGPPGPP GLMGPPGPPG LPGPKGNMGL NFQGPKGEKG EQGLQGPPGP
241     PGQISEQKRP IDVEFQKGDQ GLPGDRGPPG PPGIRGPPGP PGGEKGEKGE QGEPGKRGKP
301     GKDGENGQPG IPGLPGDPGY PGEPGRDGEK GQKGDTGPPG PPGLVIPRPG TGITIGEKGN
361     IGLPGLPGEK GERGFPGIQG PPGLPGPPGA AVMGPPGPPG FPGERGQKGD EGPPGISIPG
421     PPGLDGQPGA PGLPGPPGPA GPHIPPSDEI CEPGPPGPPG SPGDKGLQGE QGVKGDKGDT
481     CFNCIGTGIS GPPGQPGLPG LPGPPGSLGF PGQKGEKGQA GATGPKGLPG IPGAPGAPGF
541     PGSKGEPGDI LTFPGMKGDK GELGSPGAPG LPGLPGTPGQ DGLPGLPGPK GEPGGITFKG
601     ERGPPGNPGL PGLPGNIGPM GPPGFGPPGP VGEKGIQGVA GNPGQPGIPG PKGDPGQTIT
661     QPGKPGLPGN PGRDGDVGLP GDPGLPGQPG LPGIPGSKGE PGIPGIGLPG PPGPKGFPGI
721     PGPPGAPGTP GRIGLEGPPG PPGFPGPKGE PGFALPGPPG PPGLPGFKGA LGPKGDRGFP
781     GPPGPPGRTG LDGLPGPKGD VGPNGQPGPM GPPGLPGIGV QGPPGPPGIP GPIGQPGLHG
841     IPGEKGDPGP PGLDVPGPPG ERGSPGIPGA PGPIGPPGSP GLPGKAGASG FPGTKGEMGM
901     MGPPGPPGPL GIPGRSGVPG LKGDDGLQGQ PGLPGPTGEK GSKGEPGLPG PPGPMDPNLL
961     GSKGEKGEPG LPGIPGVSGP KGYQGLPGDP GQPGLSGQPG LPGPPGPKGN PGLPGQPGLI
1021    GPPGLKGTIG DMGFPGPQGV EGPPGPSGVP GQPGSPGLPG QKGDKGDPGI SSIGLPGLPG
1081    PKGEPGLPGY PGNPGIKGSV GDPGLPGLPG TPGAKGQPGL PGFPGTPGPP GPKGISGPPG
1141    NPGLPGEPGP VGGGGHPGQP GPPGEKGKPG QDGIPGPAGQ KGEPGQPGFG NPGPPGLPGL
1201    SGQKGDGGLP GIPGNPGLPG PKGEPGFHGF PGVQGPPGPP GSPGEALEGP KGNPGPQGPP
1261    GRPGPTGFQG LPGPEGPPGL PGNGGIKGEK GNPGQPGLPG LPGLKGDQGP PGLQGNPGRP
1321    GLNGMKGDPG LPGVPGFPGM KGPSGVPGSA GPEGEPGLIG PPGPPGLPGP SGQSIIIKGD
1381    AGPPGIPGQP GLKGLPGPQG PQGLPGPTGP PGDPGRNGLP GFDGAGGRKG DPGLPGQPGT
1441    RGLDGPPGPD GLQGPPGPPG TSSVAHGFLI TRHSQTTDAP QCPQGTLQVY EGFSLLYVQG
1501    NKRAHGQDLG TAGSCLRRFS TMPFMFCNIN NVCNFASRND YSYWLSTPEP MPMSMQPLKG
1561    QSIQPFISRC AVCEAPAVVI AVHSQTIQIP HCPQGWDSLW IGYSFMMHTS AGAEGSGQAL
1621    ASPGSCLEEF RSAPFIECHG RGTCNYYANS YSFWLATVDV SDMFSKPQSE TLKAGDLRTR
1681    ISRCQVCMKR T
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Figure 6. 1691 amino acids codified by the COL4A5 gene normally.

nopathy, and retinal thinning) have been reported amongst XLAS patients [19, 20].

However, not all kidney disease cases display the features of Alport syndrome, and these complications were not observed in our family members. Therefore, it is difficult to diagnose based only on clinical manifestations. These patients were clinically suspected to have XLAS because there was no male-to-male transmis-

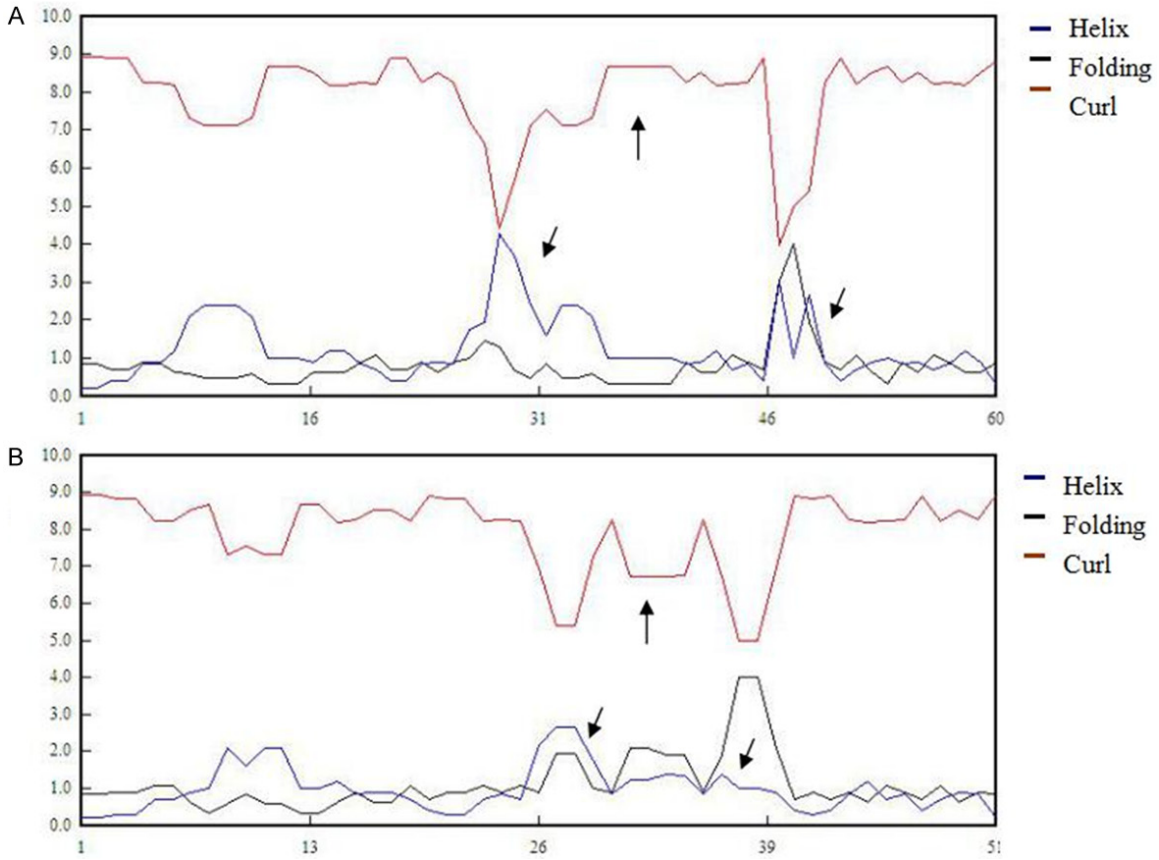

Figure 7. The secondary structure of the protein codified by the COL4A5 gene at positions 1237-1245 (A: Normal, B: The mutation in the family).

sion in this family, and the male patients were sicker than the female patients. However, the pattern of autosomal dominant inheritance cannot be excluded. Furthermore, neither hearing impairment nor ophthalmological abnormality was observed in those patients. The clinical information increased the complexity of identifying the genetic cause of glomerulopathy in the family. Thus, we performed a skin biopsy, a renal biopsy, and a direct DNA analysis in COL4A5 to further verify our hypothesis.

The diagnosis of AS is made with the demonstration of a lamellated GBM on ultrastructural examination of the renal biopsy. Although light microscopy studies typically show focal segmental glomerulosclerosis (FSGS) over the course of time, specific anomalies observed by electron microscopy in our study include thickening, splitting and fragmentation of the lamina densa [3, 21]. In addition, immunohistochemical analysis of the expression of α5 (IV) in the epidermal basement membrane and/or of α5α4α3 (IV) in the GBM are additional AS diagnostic tools [2, 3, 22, 23].

In XLAS, the collagen IV α5 chain and the α3 and α4 chains are often absent from the GBM and from the epidermal basement membrane; in autosomal recessive disease, the α3α4α5 network is absent from the GBM, but the α5 chain persists in the α5α5α6 network in the epidermis. In our study, the absence of the α5 chain from collagen type IV in the skin basement membrane suggests a diagnosis of XLAS for the family members.

A hallmark feature of XLAS patients is the age-dependent penetrance and a broad phenotypic heterogeneity; the progression of nephropathy is variable. In XLAS, genetic mutations are typically different in each family, and clinical features in males depend on the mutation characteristics and location [5, 24, 25]. Thus, a genetic mutational investigation is helpful for further making a diagnosis earlier and predict-

ing prognosis and opening a window of opportunity for early intervention. Genetic testing has been accepted as the gold standard for the diagnosis of AS and the demonstration of its mode of inheritance [22].

In our family, three patients presented with heterogeneous clinical phenotypes of glomerulopathy, while none showed any clinical features of either sensorineural hearing loss or typical *COL4A5*-related ocular abnormalities. A *COL4A5* c.499delC (p.Pro167Gln*36) mutation in exon 41, cosegregating with the disease, was identified. The deletion mutation led to a truncated protein and was absent in the 1,000 genomes project, HapMap, dbSNP, and 100 normal controls. Our clinical and genetic data also support an X-linked inheritance form of AS in this family. The mutation may decrease the mechanical stability of the glomerular basement membrane (GBM) because of the secondary structural changes of the protein coded by *COL4A5*.

A female with X-linked disease is usually advised that, on average, half her children will inherit the mutation, and half her sons will develop renal failure, but her daughters have only a 15% risk of renal failure by the age of 60. By contrast, all the offspring of a woman with two COL4A5 mutations will inherit a mutation, and all her sons will eventually develop end-stage kidney disease [25].

The different clinical manifestations amongst family members bearing the same mutation in our pedigree may be due to random X chromosome inactivation [26] and genetic background and environmental factors. Further studies on the genetic and epigenetic factors modifying the expression and function of the COL4A5 gene may help us to better understand the molecular basis of AS [27]. Animal models with genetic deficiencies may provide new insight into mechanism research, diagnosis, and target therapy of AS in humans.

Furthermore, pathogenic deep intronic mutations have also been reported but cannot be covered by Sanger sequencing. This could be considered a limitation in our study, and newer sequencing techniques (e.g., targeted next generation sequencing) will allow the analysis of these intronic mutations. Additionally, digenic mutations may have been missed by Sanger sequencing, which would not be overlooked by NGS [27-29]. Children with AS can usually be diagnosed with the disorder in their first decade of life, at which time they are typically oligosymptomatic, with mild haematuria and low-grade proteinuria. As the disease progresses, patients gradually develop severe proteinuria and progressive renal failure. In our family, all male and female carriers were asymptomatic, which may be due to their young ages.

Conclusions

In our study, we identified a novel deletion mutation, c.499delC (p.Pro167Gln55*36), in the *COL4A5* gene, which may be responsible for AS in this family. The discovery broadens the genotypic spectrum of *COL4A5* mutations associated with AS and has implications for genetic diagnosis, therapy, and genetic counselling of this family.

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Disclosure of conflict of interest

None.

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Supplementary Table 1. The primers designed by primer 5 programme

Number	Fragment	Primer sequences	Product length (bp)
1	Exon 1	F 5' GGGGAAGAGAGGGAGGAAAG 3'	497
		R 5' CCCTAGTCAACGCCAAAAGG 3'	
2	Exon 2	F 5' TGGATTGTTGATTTCAGTTGAGC 3'	475
		R 5' CCCAAGAGGCTCATGTTCTAT 3'	
3	Exon 3	F 5' TGAATCTCAACCATGCCTGTG 3'	299
		R 5' ACTCCCCGTCATTCCATTGT 3'	
4	Exon 4	F 5' ACCTCAGTGCTTTCAGTCTCA 3'	582
		R 5' CTTCTGGACCTGAAGACACTAT 3'	
5	Exon 5~6	F 5' ATCCACGAATTTGCCTGCTC 3'	576
		R 5' GCCAAGACCTGAAGACACTAT 3'	
6	Exon 7~8	F 5' TCTGGCATGTTTCAAAATGGC 3'	604
		R 5' TTGAAGTTGCCAGCTTTCCT 3'	
7	Exon 9	F 5' AGTGTACTCTGGCCACTTCC 3'	339
		R 5' ACTAAAGGTTGAGGGATTGTTGT 3'	
8	Exon 10	F 5' GTCGCAGTGAGTTGAGATCA 3'	384
		R 5' TGGAGCCATTACTTAGAATGCA 3'	
9	Exon 11~13	F 5' CTACCTCTGCCCATCCCAG 3'	699
		R 5' TCATTGACTTCCTCCCTACTTAC 3'	
10	Exon 14~16	F 5' GCTCTAACCATGTTGCTCCA 3'	674
		R 5' ATGTCTCCAGTTTTGCAGGG 3'	
11	Exon 17	F 5' ACACTCCTTGAATTGCTGCA 3'	284
		R 5' AGCCTGGGAGAAGAAAAGGA 3'	
12	Exon 18	F 5' TCACAGGTTAGGCTTAGCTCA 3'	292
		R 5' AGGGAGTATGCGATTAAGGC 3'	
13	Exon 19	F 5' GCTCCCATAATGTTTTCAGGAGA 3'	297
		R 5' TGCTGACCATGGAACTGTTACT 3'	
14	Exon 20	F 5' TTTCCTACCCTCAAACTGTTACT 3'	465
		R 5' CGCAACAAATGAGGCCAACC 3'	
15	Exon 21	F 5' GCTTGTCAGGCTTTCTTTTGTT 3'	250
		R 5' GGCTTAGGTGTGATGGAGGA 3'	
16	Exon 22	F 5' TGCTGTCCCTTAGTGCTAACA 3'	243
		R 5' ACCTCAACAAAATCTAGCCAAAA 3'	
17	Exon 23	F 5' TCTGTATGAGCTTTGTCAGGAGT 3'	363
		R 5' ACCCCTTCAACCCATCCAAT 3'	
18	Exon 24	F 5' GGGTTGAAGGGGTAAACTGG 3'	397
		R 5' TCTCAGCATCAGTCCCATCC 3'	
19	Exon 25	F 5' TGGCTATATCCTTTCCCCAGT 3'	338
		R 5' AGGAAGCCATGAGTAGCCAA 3'	
20	Exon 26~27	F 5' TTATACATCTTGAATCATGGAAGTC 3'	867
		R 5' CCTACTCTGCCTGCTGCTACCCA 3'	
21	Exon 28	F 5' GAATGTGGGTTTGGGAATTTGAA 3'	243
		R 5' AGCTAGAAATAAGGAAGGTGGGA 3'	
22	Exon 29	F 5' CGGCATTAAATTCTCTGTGGC 3'	261
		R 5' GTTGAGATGCAGTGACAGCC 3'	
23	Exon 30	F 5' TGCTGAATGAATGCCCAGTT 3'	385
		R 5' CTGTGGCTTGTACTTATCCACT 3'	
24	Exon 31	F 5' TTGTGTGCATGATGTCAAAAGTA 3'	387
		R 5' TGGGAATTATCTACCAGAGTCGT 3'	

25	Exon 32	F 5’ AACAGTGCCTTACGTCCAAC 3’ R 5’ ACATACGTCTGGCAGCAAAC 3’	235
26	Exon 33	F 5’ ATGTTTTGAGTGGCCAGTGG 3’ R 5’ AGTGCCTTTGTTGGTGAATTCT 3’	373
27	Exon 34	F 5’ TGAGTAGCTTGCTTTGCCAA 3’ R 5’ ATCCAGAATTCAGTGTCAGCT 3’	232
28	Exon 35	F 5’ TCCCATGAAACCAGACAACC 3’ R 5’ TCAACACCAGCCTCTTTTCTG 3’	246
29	Exon 36	F 5’ AATTGTGTTTGTGAGGCTCATT 3’ R 5’ GACTGGCCACAAACCCTAAA 3’	468
30	Exon 37	F 5’ TGAAGCCTAACTCACAGAACTG 3’ R 5’ AGCTGCTCTGTGATACTGGT 3’	364
31	Exon 38	F 5’ TCTTCACTGTTTCTATGCTAGCA 3’ R 5’ GCTGAACATGATTTGACTTTCCC 3’	267
32	Exon 39	F 5’ GGGAGCATATGGAAGTAAAAGGG 3’ R 5’ AGGGGAAAGTGTGTGGTAGC 3’	294
33	Exon 40	F 5’ TGCTGCACCTAATGAAAACTT 3’ R 5’ AGGTTTGAGTTTTCTGGGTTCT 3’	283
34	Exon 41	F 5’ TCCTCCAGCCATCTTGAAACT 3’ R 5’ AACCTGCCAGCAAAACGAAA 3’	595
35	Exon 42	F 5’ TTGGGCCTTGGTGAACTTTG 3’ R 5’ ACACTGGGTTCTACAAGTTCCT 3’	207
36	Exon 43	F 5’ ACTGTGTGATGTCTGATACTGC 3’ R 5’ ACCCCAGCTTGAGGATAACA 3’	202
37	Exon 44	F 5’ TGTTGTGTGGGTTTTATTCTGCA 3’ R 5’ TTTAATTCGCCAGTGAGCCA 3’	385
38	Exon 45~46	F 5’ CACCTCAGTTAGCCATGGGA 3’ R 5’ CGATGGTCTGGCTGAAAGAC 3’	696
39	Exon 47	F 5’ ACTTAGGTCACTTTGGCTTCC 3’ R 5’ CTGCATTGGTACCTGGTAAGC 3’	214
40	Exon 48	F 5’ TGAATTCAGTGTCTCGAGAACC 3’ R 5’ TTTCCCTCCACCAACAGCAT 3’	229
41	Exon 49	F 5’ AGCCCATGATATCTGACAATGC 3’ R 5’ CCAAGGCTACTCTAGAACCCA 3’	443
42	Exon 50	F 5’ TGAAAGGCTGGCAAGTTTCC 3’ R 5’ AGCTAACTAACTGGGGATTGGT 3’	335
43	Exon 51	F 5’ GCAGGATGGCTACTTCTCAC 3’ R 5’ AGATGGAATGGACAGTCAGCT 3’	234