Identification of four novel mutations in the *COL4A5* gene identified in Chinese patients with X-linked Alport syndrome

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Abstract. Alport syndrome (AS) is an inherited progressive nephropathy caused by mutations in one or two of the type IV collagen novel chains (α3, α4 and α5), which are encoded by *COL4A3*, *COL4A4* and *COL4A5*, respectively. To date, three genetic forms of AS have been reported, including X-linked AS, autosomal recessive AS, and autosomal dominant AS, and ~80% of patients have X-linked AS caused by mutations in *COL4A5*. In the present study, four novel and one previously reported *COL4A5* mutations were identified using targeted next-generation sequencing in Chinese patients suspected of having AS. The results were confirmed by Sanger sequencing, which revealed two novel missense mutations resulting in the substitution of various glycine residues in a collagenous domain containing Gly-X-Y triplet sequence repeats [c.4198G>C, p.(Gly1400Arg) and c.3428G>T, p.(Gly1143Val)], a previously reported missense mutation [c.3071G>A, p.(Gly1024Glu)], a splice site mutation (c.2146+2T>A) and one frameshift mutation [c.1810delC (p.Thr605Ilefs*13)]. After analyzing the affected family members, it was shown that the identified mutations were associated with severe clinical phenotypes. These results broaden the known spectrum of mutations of the *COL4A5* gene associated with AS and may have implications for genetic diagnosis, therapy and genetic counseling of affected families.

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Introduction

Alport Syndrome (AS) is an inherited nephropathy caused by mutations in one or two of the type IV collagen chains (α3, α4 and α5) (1-3). AS is characterized by persistent microscopic hematuria starting during infancy, and eventually leading to either progressive nephritis or end-stage renal disease (ESRD), along with extrarenal abnormalities, such as sensorineural deafness and ocular anomalies (4). In ~80% of AS cases, patients exhibit X-linked inheritance with mutations in the *COL4A5* gene. Autosomal recessive inheritance due to biallelic mutations in *COL4A3* or *COL4A4* are found in ~15% of patients, and the remaining 5% of cases are due to autosomal dominant inheritance, due to heterozygous mutations in *COL4A3* or *COL4A4* (5).

Patients with AS display a wide array of phenotypic variability, ranging from progressive renal disease to isolated hematuria (6). The severity of renal manifestations in X-linked AS (XLAS) differs between males and females (6). All male patients with XLAS develop proteinuria, and eventually progress to renal insufficiency (7). However, female patients have milder and more variable clinical presentations, ranging from isolated hematuria to ESRD, with later onset and slower disease progression (8). Electron microscopy analysis of renal biopsies suggested that the majority of patients exhibit structural alterations in the glomerular basement membrane (GBM). The earliest change in AS is diffuse thinning of the GBM (9). Frequently, the diagnostic ultrastructural changes in the GBM for adult males are a basket-weave-like change, whereas the predominant alteration observed in children and female patients with XLAS is diffuse thinning of the GBM (9). Therefore, in the absence of a family history of either hematuria or ESRD, it may be difficult to perform a pathological diagnosis for female patients with isolated hematuria (4,10,11).

Genetic testing is an effective tool for clinical diagnosis and prognosis of AS, and to support counseling of affected patients. In the present study, targeted next-generation sequencing (NGS) was used to identify four novel and one reported *COL4A5* mutations in Chinese patients suspected of having AS.

Materials and methods

Patients and ethics. All subjects provided signed informed consent forms for participation in the present study; consent from the probands IID2, IID3 and IID4 (all <18 years) was obtained from their parents. The present study was approved by the Zhengzhou University Ethics Committee (Zhengzhou, China).

Patients and families. In total, 5 families were included in the present study who were recruited between June 2018 and May 2019. The age of patients ranged from 5-26 years old and four male probands and one female proband were included. Clinical diagnosis of patients was performed by a nephrologist based on clinical manifestations and biochemical analysis, such as hematuria, proteinuria and high creatinine levels. A brief clinical summary of the probands are presented in Table I.

Samples and DNA extraction. Genomic DNA was extracted from EDTA peripheral blood samples using Lab-Aid® 824 DNA extraction kit according to the manufacturer's protocol (Xiamen Zeesan Biotech Co., Ltd.).

Custom panel design. A custom panel was designed for the *COL4A3*, *COL4A4* and *COL4A5* genes using the Ion AmpliSeq™ designer software version 7.4.2 (Thermo Fisher Scientific, Inc.) to perform mutational screening of patients suspected of having AS. The coding regions and all the flanking introns up to 50 bp were targeted. The 3' and 5' untranslated regions were not included in the panel design. Details of the methodology have been described previously (12).

Ion torrent personal genome machine (PGM) sequencing. Library preparation was performed by amplifying 10 ng genomic DNA, using the Ion AmpliSeq™ Library kit 2.0 (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Libraries were purified using the Agencourt® AMPure® XP system and quantified using the Qubit® dsDNA HS assay kit (Invitrogen; Thermo Fisher Scientific, Inc.) and clonally amplified by emulsion PCR using the Ion OneTouch™ 2 system (Ion PGM™ Template Hi-Q™ view OT2 200 kit; Thermo Fisher Scientific, Inc.) all according to the manufacturer's protocol. The spheres were loaded on to a 316™ v2 chip and sequenced on the Ion Torrent PGM, using the Ion PGM™ Hi-Q™ view Sequencing 200 kit v2. Post-run analysis was performed using Torrent Suite™ version 5.0.4 (Thermo Fisher Scientific, Inc.). Coverage assessment was performed using the 'coverage Analysis' plug-in which provides information regarding the amplicons read coverage, and variants were called using the 'variant Caller' plug-in (12).

Results

In total, 5 probands were included in the present study. The eye examinations showed there were no abnormalities in all patients. A total of three missense mutations, one splice site mutation and one frameshift mutation were identified (Table II). Of the 5 patients, 4 patients had novel mutations which had not been reported previously, to the best of our knowledge. These candidate mutations were validated by Sanger sequencing (Fig. 1). The predicted clinical significance of these mutations, which were assessed using the criteria of clinical significance based on the American College of Medical Genetics guidelines (13), are listed in Table II.

In AS patient IID 1, systemic edema was first noted 6 years ago without an obvious cause, alongside proteinuria, but without hematuria or hypertension. The disease was diagnosed as nephrotic syndrome. Subsequently, 3 years ago, facial edema and fatigue appeared again, accompanied by intermittent headaches, with a highest recorded blood pressure of 180/100 mmHg, urinary protein 3+ and blood creatinine levels of 200 μmol/l. This was treated with hormone therapy. Facial edema and weakness appeared again 1 year prior to this study, with a highest recorded blood pressure of 200/120 mmHg, hemoglobin levels of 66.0 g/l, urinary protein 3+ and point albumin levels of 182.56 mg/mmol. The urea level was 49.03 mmol/l, the creatinine levels were 1,115 μmol/l and the uric acid level was 514 μmol/l. They were diagnosed with CKD-5 with sensorineural hearing loss, and received regular hemodialysis treatment for 1 year. The patient progressed to the uremia stage and received an allograft kidney transplant. Renal biopsy indicated that there was irregular thickness and wrinkling of the GBM. A hemizygous spontaneous variation of the *COL4A5* gene [c.3071G>A, p.(Gly1024Glu)] was detected by NGS. The patients 6-year-old daughter presented with intermittent hematuria and proteinuria, and was found to possess the same heterozygous mutation [c.3071G>A, p.(Gly1024Glu)] (Fig. 1A).

In AS patient IID 2, urine foaming and hematuria were observed for >7 months, with blood creatinine levels of 61 μmol/l, heterogeneous small red blood cells, blood pressure of 115/70 mmHg and urinary protein 3+. The renal biopsy showed that the GBM exhibited irregular thickness and wrinkling. A hemizygous variation of the *COL4A5* gene [c.4198G>C, p.(Gly1400Arg)] was identified by NGS, which was inherited from the patient's mother, whom exhibited intermittent microscopic hematuria (Fig. 1B).

The pediatric AS patient IID 3 had gross hematuria with no obvious cause 22 days prior to admission. There was intermittent frequent pain and itchiness when urinating, urinary protein 2+ and blood creatinine levels of 158 μmol/l. The renal biopsy showed a GBM with an irregular thickness and wrinkling. A hemizygous variation of the *COL4A5* gene [c.3428G>T, p.(Gly1143Val)] was detected by NGS, which was inherited from the patient's mother, who exhibited intermittent microscopic hematuria (Fig. 1C).

AS patient IID 4 (IV-2 in Fig. 1D) was a 7-year-old male with first onset of gross hematuria at the age of 4. By the age of 7, he had already developed proteinuria, and biochemical analysis indicated high plasma creatinine levels. His mother showed intermittent microhematuria. IV-1 progressed to chronic kidney disease and the patient received renal dialysis. IV-3 exhibited hematuria and proteinuria. III-2 showed intermittent microhematuria and hypertension, whereas III-6 only presented with microhematuria. II-2 and II-3 progressed to ESRD at the age 25 and 27 years, respectively. They both received renal transplants, and both exhibited sensorineural deafness (Fig. 1D). A novel splicing mutation (c.2146+2T>A) at the splice donor site at the boundary between exon 27/intron 27

Table I. Clinical and pathological characteristics of the patients.

IID	Sex	Age, years	Hearing loss	Renal biopsy		Family history
				EM	α3/α5	
IID1	Male	26	Mild	BWC	A/A	P
IID2	Male	15	Normal	BWC	M/A	P
IID3	Male	6	Normal	BWC	A/A	P
IID4	Male	10	Normal	BWC	A/A	P
IID5	Female	25	Normal	TBM	Nor/M	N

IID, individual ID; EM, electron microscope; BWC, basket-weave change; TBM, thin basement membrane; P, positive; N, negative; M, mosaic patchy loss of staining for the collagen α3, α4 and α5 (type IV) chains in the glomerular basement membrane; A, absence; Nor, normal.

Table II. Mutations detected in the *COL4A5* gene.

Sample	Zygosity	Type of mutation	cHGVS	pHGVS	Clinical significance[a]	Novelty
IID1	Hemizygous	Missense	c.3071G>A	p.(Gly1024Glu)	LP	Previously reported
IID2	Hemizygous	Missense	c.4198G>C	p.(Gly1400Arg)	VUS	Novel
IID3	Hemizygous	Missense	c.3428G>T	p.(Gly1143Val)	LP	Previously reported
IID4	Hemizygous	Missense	c.2146+2T>A	splicing	LP	Novel
IID5	Heterozygous	Frameshift	c.1810delC	p.(Thr605Ilefs*13)	LP	Novel

[a]Based on American College of Medical Genetics criteria. IID, individual ID; LP, likely pathogenic; VUS, variant uncertain significance; HVGS, Human Genome Variation Society.

in *COL4A5* in the proband was identified. The candidate mutation was validated by Sanger sequencing and it was predicted to mutate a normal splice site according to the Human Splicing Finder (umd.be/HSF3/index.html). The proband's mother was heterozygous for the c.2146+2T>A mutation, whereas the healthy family member, III-7, did not have a mutation at this site (Fig. 1D).

AS patient IID 5 was a 25-year-old female with gross hematuria, urinary protein 2+, blood creatinine 100 μmol/l and point albumin 19.63 mg/mmol. Her urea levels were 69.03 mmol/l, the creatinine levels were 895 μmol/l and uric acid levels were 324 μmol/l. The renal biopsy showed lesions in the GBM. A novel spontaneous mutation, c.1810delC (p.Thr605Ilefs*13), was identified in *COL4A5* in the proband (Fig. 1E).

Discussion

The heterotrimer of α3, α4 and α5 chains serve an important role in the structure and function of the basement membrane of the glomeruli, cochlea and eyes (14). A collagenous domain of the α5 chain contains Gly-X-Y triplet sequence repeats (15). A missense mutation replacing the glycine residue in a Gly-X-Y repeat accounts for ~30% of *COL4A5* mutations in XLAS. Glycine substitution in the collagenous domain is hypothesized to introduce kinks in the protein, thus interfering with the proper folding of the collagen triple helix (14,16). It has been reported that glycine substitutions in the α5 chain (type IV) result in different structural changes to the GBM, which are associated with the clinical phenotype of AS (14). Thus far, three types of phenotype have been associated with typical XLAS based on the time taken to progress to ESRD, which is used to classify the severity (severe type, intermediate type and moderate type). The severe type progresses to ERSD at ~20 years of age (early-onset ESRD), with a high incidence of sensorineural deafness and ocular changes, caused by large rearrangements, premature stop, frameshift, donor splice and missense mutations in the NC1 domain. Patients with intermediate type progress to ESRD at ~26 years of age, due to non-Gly-X-Y missense or Gly-X-Y mutations in exons 21-47. In the moderate type, patients progress to ESRD after ~30 years (late-onset ESRD), which is accompanied by a lower incidence of sensorineural deafness and ocular changes, due to Gly-X-Y mutations in exons 1-20 (17-19).

Due to the large size of *COL4A5* gene, there are no known mutational hot spots, to the best of our knowledge. To date, 1,119 mutations in *COL4A5* have been recorded according to the Human Gene Mutation Database (HGMD® Professional version 2019.2; portal.biobase-international.com/hgmd/pro/start.php), including missense, nonsense, deletion or splicing mutations, as well as complex rearrangements. In the present study, three missense mutations [c.3071G>A p.(Gly1024Glu), c.4198G>C p.(Gly1400Arg) and c.3428G>T p.(Gly1143Val)] were identified which resulted in the substitution of glycine located in exons 21-47. The proband with the c.3071G>A p.(Gly1024Glu) mutation in family 1 progressed to the uremia stage at the age of 26 and developed sensorineural

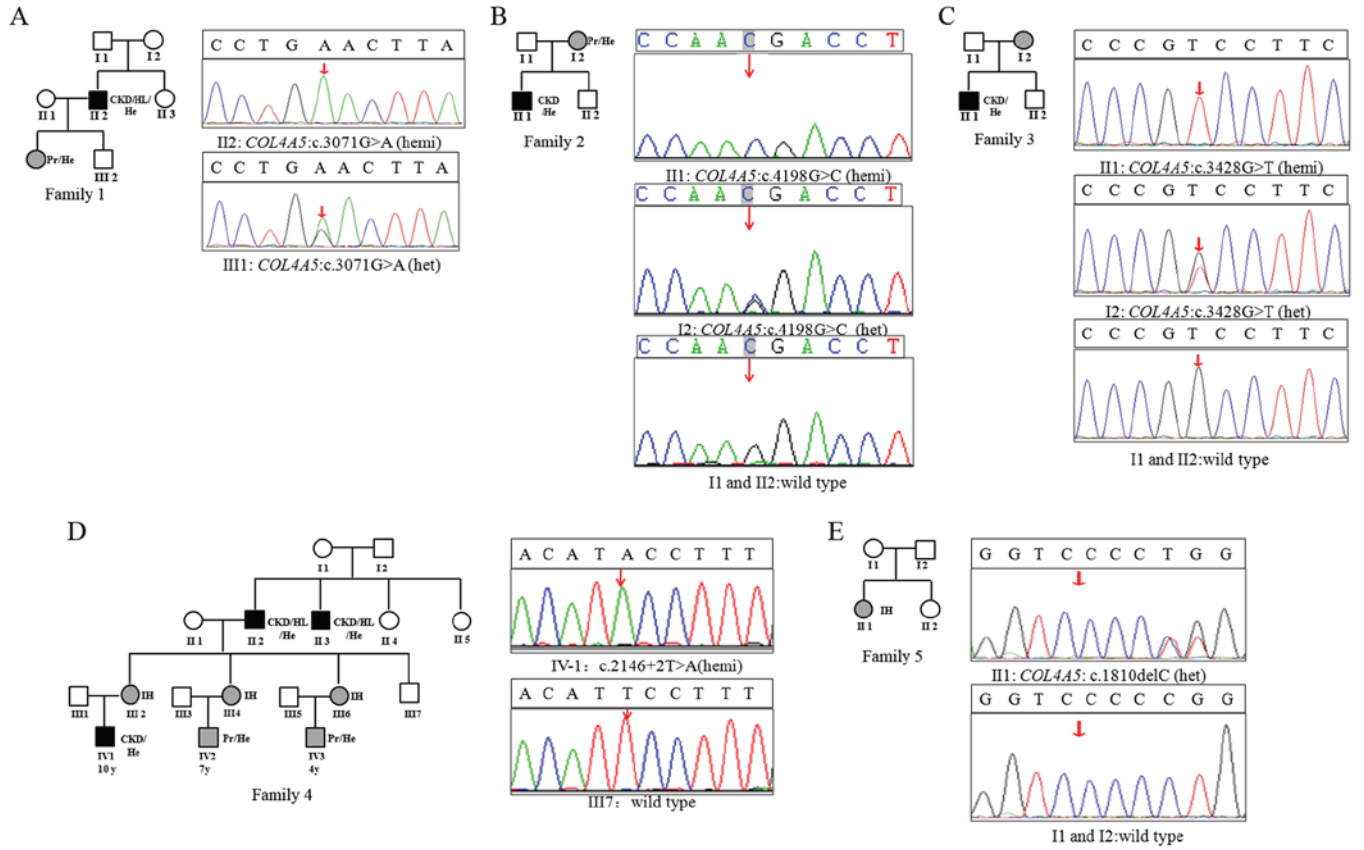

Figure 1. Pedigrees of the families with X-linked Alport syndrome and sequencing analysis of candidate *COL4A5* variants. (A-E) Left panel: The pedigrees of family 1-5; (A-E) Right panel: Sequencing analysis of candidate *COL4A5* variants. Dark colours indicate patients with severe clinical symptoms who CKD. Light colours indicate patients with mild clinical symptoms who only exhibited showing proteinuria and hematuria. No colours indicate healthy patients. HL, hearing loss; CKD, chronic kidney disease; Pr, proteinuria; He, hematuria; hemi, hemizygous; het, heterozygous.

hearing loss. His daughter (6 years) showed intermittent hematuria and proteinuria. It has been previously reported that a patient suffering from AS had the same mutation (20). These findings suggest that the mutation may be associated with severe clinical phenotypes and an earlier age of onset. The probands IID2 (15 years) and IID3 (6 years) both exhibited renal insufficiency due to the mutations c.4198G>C, p.(Gly1400Arg) and c.3428G>T, p.(Gly1143Val) inherited from their mothers, who had intermittent microscopic hematuria. It has been previously reported that a patient suffering from AS possessed the mutation c.3428G>A, p.(Gly1143Asp) in the *COL4A5* gene (21). Considering their young age, further follow-up is required to fully assess their clinical symptoms. Based on a previous report of intermediate type XLAS due to non-Gly-X-Y missense and Gly-X-Y mutations exons in 21-47 (18), it was hypothesized that the mutations [c.4198G>C, p.(Gly1400Arg) and c.3428G>T, p.(Gly1143Val)] were also associated with severe clinical phenotypes.

A novel splicing mutation, c.2146+2T>A was identified in the *COL4A5* gene in the proband IID4. The same mutation was subsequently found in all 7 affected family members (II-2, II-3, III-2, III-4, III-6, IV-1 and IV-3), and was absent in the unaffected members. This variant has not been reported in any public databases. Based on the phenotypes of affected family members, it was concluded that the splicing site mutation c.2146+2T>A was also associated with severe clinical phenotypes.

Due to the relatively milder phenotypes in female patients with X-linked AS, it is may be difficult to perform a pathological diagnosis for females with isolated hematuria. In the present study, a novel spontaneous mutation [c.1810delC (p.Thr605Ilefs*13)] in *COL4A5* in a 25-year-old female patient (IID5) was found, who exhibited hematuria and proteinuria, together with lesions in the GBM, but did not have a family history of related diseases. This case highlights NGS as an effective method for obtaining genetic sequencing information in female patients with XLAS.

In conclusion, four novel mutations of the *COL4A5* gene were identified and were shown to be associated with AS. The present study broadens the known spectrum of mutations of the *COL4A5* gene and may have implications for genetic diagnosis, therapy and genetic counseling of affected families.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

XZ and XK designed the present study. XS, CC and LL performed the experiments. GZ and JZ collected the data. XS and CW analyzed the data. XZ and XK wrote the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

All subjects provided signed informed consent forms for participation in the present study; consent from the probands IID2, IID3 and IID4 (all <18 years) was obtained from their parents. The present study was approved by the Zhengzhou University Ethics Committee (Zhengzhou, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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