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Abnormal mRNA Splicing Effect of *COL4A3* to *COL4A5* Unclassified Variants

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Introduction: Genetic diagnosis of Alport syndrome (AS), which results from pathogenic variants in *COL4A3*, *COL4A4*, or *COL4A5* genes, is hindered by large numbers of unclassified variants detected using next-generation sequencing (NGS). We examined the impact on splicing of variants of uncertain significance in *COL4A3* to *COL4A5*.

Methods: Nine unrelated patients with clinical diagnosis or suspicion of AS were enrolled according to the criteria. Their clinical and genetic data were collected. Blood and urine samples were obtained from the patients and their family members. Sanger sequencing was used to confirm the 9 *COL4A3* to *COL4A5* unclassified variants identified by NGS. *COL4A3* to *COL4A5* mRNAs from urine were analyzed using targeted reverse transcription polymerase chain reaction and direct sequencing.

Results: Nine *COL4A3* to *COL4A5* unclassified variants were found to alter mRNAs splicing. Skipping of an exon or an exon fragment was induced by variants *COL4A3* c.828+5G>A; *COL4A4* c.3506-13_3528del; and *COL4A5* c.451A>G (p. [Ile151Val]), c.2042-9 T>G, c.2689 G>C (p. [Glu897Gln]) and c.1033-10_1033-2delGGTAATAAA. Retention of an intron fragment was caused by variants *COL4A3* c.3211-30G > T, and *COL4A5* c.4316-20T>A and c.1033-10 G>A, respectively. The 9 families in this study obtained genetic diagnosis of AS, including 3 with autosomal recessive AS and 6 with X-linked AS.

Conclusions: Our findings demonstrate that urine mRNA analysis facilitates the identification of abnormal splicing of unclassified variants in Alport genes, which provides evidence of routine use of RNA analysis to improve genetic diagnosis of AS.

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AS is an inherited kidney disease characterized by hematuria, proteinuria, and progressive kidney failure frequently accompanied by hearing loss and ocular lesions.[1,2] It is caused by pathogenic variants in *COL4A3*, *COL4A4*, or *COL4A5* genes, which encode the type IV collagen α3, α4, and α5 chains, respectively.[3-5] The triple helical isoform of α3-α4-α5 (IV) is a major structural component of mature glomerular basement membrane (GBM), which would be impaired because of pathogenic variants in any of the 3 Alport genes.[6]

Pathogenic variants in *COL4A5* gene cause X-Linked AS (XLAS).[7] It is well known that there is a strong genotype and phenotype correlation in males with XLAS. In a European population, it was reported that the risk of developing end-stage kidney disease (ESKD) before the age of 30 years in males with large deletions, nonsense mutations, or small mutations changing the reading frame in *COL4A5* gene was 90%, whereas the same risk was 70% in males with splicing mutations, and 50% in males with missense mutations.[8] Recently, in a Chinese cohort, the median age of ESKD in males with XLAS was reported to be 39 and 22 years for nontruncating and truncating mutations in *COL4A5* gene, respectively.[9] Furthermore, the frequency of hearing loss and effect of renin-angiotensin-aldosterone system blockers in males with XLAS with non-truncating and truncating mutations were significantly different. Females with XLAS demonstrated widely variable disease outcomes.[10,11] A systematic review of pathogenic *COL4A5* variants and proteinuria in females with XLAS found that proteinuria correlated with a more severe genotype.[12]

Autosomal recessive AS (ARAS) is due to pathogenic variants in both alleles of either *COL4A3* or *COL4A4* gene.[13] Females and males with ARAS are equally affected. The median onset age of ESKD is about 21years.[14] The phenotype of ARAS correlates with the

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number of missense variants in *COL4A3* or *COL4A4* gene. Patients without missense variants had more severe outcomes than those who had 1 or 2 missense variants.[15] Autosomal dominant AS is caused by pathogenic heterozygous variants in *COL4A3* or *COL4A4* gene.[16,17] There is a small number of patients with digenic AS because of pathogenic variants in 2 of the *COL4A3* to *COL4A5* genes. These patients have highly variable clinical presentations.[18,19] A recent systematic literature review for patients with pathogenic heterozygous variants in *COL4A3* or *COL4A4* gene found that the median age at kidney failure was 55 years for individuals with missense variants, and 47 years for those with nonmissense variants resulting in premature termination of translation.[20]

The above-mentioned studies indicate that analysis of genotype-phenotype correlations in AS can help to predict the risk and onset age of ESKD. However, variants of unknown significance (VUSs) in Alport genes pose a challenge for genetic diagnosis and subsequent analysis of genotype-phenotype correlations. Published studies demonstrate that abnormal splicing, which accounts for about 13% to 25% of pathogenic variants in *COL4A3* to *COL4A5* genes, could be caused by canonical splice site variants and noncanonical splice site variants such as deep intronic changes and substitutions in exons.[21,22] Therefore, it is necessary to determine the effect on transcripts of *COL4A3* to *COL4A5* VUSs to detect novel possible splicing variants. Analysis of *COL4A3* to *COL4A5* mRNA from cultured fibroblasts, hair roots, urine-derived cells, or *in vitro* minigene assay were the procedures reported.[23-26] Our recent study showed that analysis of urine *COL4A3* to *COL4A5* mRNAs facilitates the identification of deep intronic variants in patients with AS with negative results of NGS; thus, analyzing urine for *COL4A3* to *COL4A5* mRNA was suggested as the preferred method for patients with genetically unresolved AS.[27]

In this study, via analysis of urine *COL4A3* to *COL4A5* mRNAs, we evaluated the impact on abnormal splicing of 9 VUSs in either noncanonical splice site or in exons of *COL4A3* to *COL4A5* genes detected by NGS in 9 families with clinical diagnosis or suspicion of AS.

METHODS

Ethical Considerations

All procedures were reviewed and approved by the ethical committee of Peking University First Hospital (2020[72]), 1179]), and informed consent was obtained from the participants or their parents.

Patients and Inclusion Criteria

Patients were diagnosed or suspected of AS according to the following criteria: (i) glomerular hematuria, (ii) family history of hematuria or kidney failure without another documented kidney disease, (iii) lack of or discontinuous staining of a5 (IV) chain in epidermal basement membrane or GBM, (iv) the typical GBM ultrastructural lesions of AS (irregular thinning, thickening with splitting, and lamellation), (v) *COL4A5* pathogenic variants, (vi) *COL4A3* or *COL4A4* homozygous or compound heterozygous pathogenic variants. AS was suspected in individuals with criteria (i) and (ii) and was diagnosed with criteria (i) and 1 of (iii) to (vi).

Patients diagnosed or suspected of AS in the Pediatric Department of Peking University First Hospital from August 2020 to December 2021 were enrolled in this study according to the following 2 criteria: (i) identifying *COL4A3* to *COL4A5* VUSs using proband-only whole-exome sequencing (WES); (ii) blood and urine samples from the patients or their family members were obtained for further analysis.

The clinical data, including gender, age, renal and extrarenal manifestations, renal histopathology results, α5 (Ⅳ) expression results, and gene variants detected by WES were collected from the online registry of pediatric hereditary kidney diseases in China (http://chkd.tiamal.com/).

Genomic DNA Analyses

Genomic DNA samples from 7 probands and 13 family members were extracted from peripheral blood lymphocytes. All *COL4A3* to *COL4A5* VUSs detected with WES were confirmed in the probands and their family members using Sanger sequencing. The pathogenicity of these variants was assessed based on the American College of Medical Genetics and Genomics standards[28] and the expert consensus guidelines for the genetic diagnosis of AS.[29]

Urine mRNA Analyses

Urine samples from 6 probands and 3 mothers were collected and analyzed. Urine sample collection, mRNA extraction, and *COL4A3*–*COL4A5* mRNAs analyses were performed as described previously.[27]

RESULTS

Clinical Features

There were 40 probands who were diagnosed or suspected of AS in our department from August 2020 to December 2021. WES was performed in all of them and noncanonical splicing variants were detected in 11 patients (11/40, 27.5%). Blood and urine samples from

Table 1. Clinical characteristics and gene variants detected by whole-exome sequencing in 9 probands in this study

Proband	Gender	Age at onset (yr)	Urine analysis	Serum creatinine (umol/l)	EM findings of renal biopsy	α5(IV) expression	Family History	Gene variants (zygosity, segregation)	Location	Mutation Taster	SpliceAI	ACMG	Reference
Autosomal recessive Alport syndrome													
1	Female	10	HU, PU	51.1	AS	ND	-	COL4A3 c.2041delA, p.(Lys681Asnfs*66), (het, f)	Exon 28	DC	polymorphism	P (PVS1 PM2 PP4)	Novel
								COL4A3 c.3211-30G>T (het, m)	Intron 37	DC	D (acceptor gain 0.44)	VUS (PM2 PM3 PP4)	Novel
2	Female	3.25	HU, PU	31.6	MsPGN with normal GBM (at age of 3.7 yr)	ND	Mother (HU)	COL4A3 c.4793T>G, r.4463_5013del, p. Gly1488_His1670del, (het, f)	Exon 51	DC	polymorphism	P (PVS1 PM2 PP3 PP5)	[22]
								COL4A3 c.828+5G>A (het, m)	Intron 14	DC	D (donor loss 0.83)	VUS (PM2 PM3 PP4)	Novel
3	Female	1	HU, PU	28.5	ND	ND	Sister (HU); Mother (HU); Father (HU, PU)	COL4A4 c.3178G>A, p.(Gly1060Arg), (het, f)	Exon 34	DC	Polymorphism (acceptor loss 0.15)	LP (PM1 PM2 PP1 PP4)	Novel
								COL4A4 c.3506-13_3528del (het, m)	Intron 37-Exon 38	DC	-	VUS (PM2 PP4)	Novel
X-linked Alport syndrome													
4	Male	15	HU, PU	136.9	AS	ND	-	COL4A5 c.4316-20T>A (hemi, de novo)	Intron 48	polymorphism	D (acceptor loss 0.45)	VUS (PM2 PP4)	Novel
5	Male	5	HU, PU	32.7	ND	Negative (skin)	Mother (HU, PU); Maternal uncle (ESKD)	COL4A5 c.1033-10G>A (hemi, m)	Intron 18	DC	D (acceptor loss 0.91)	VUS (PM2 PP1 PP3 PP4)	Novel
6	Female	33	HU	ND	ND	ND	Brother (ESKD); Mother (ESKD)	COL4A5 c.451A>G, p.(Ile151Val), (het, ND)	Exon 8	polymorphism	polymorphism	VUS (PM2 PP4)	Novel
7	Female	6	HU	ND	AS	Mosaic (skin)	-	COL4A5 c.1033-10_1033-2 (het, de novo)	Intron 18	DC	-	VUS (PM2 PP3 PP4)	Novel
8	Male	1.5	HU	20	ND	ND	Mother (HU)	COL4A5 c.2042-9 T>G (hemi, m)	Intron 26	DC	polymorphism	VUS (PM2 PP1 PP4)	Novel
9	Male	3	HU, PU	ND	AS	Negative (kidney)	Mother (HU)	COL4A5 c.2689 G>C, p.(Glu897Gln), (hemi, m)	Exon 32	DC	polymorphism	VUS (PM2 PP1 PP4)	Novel

ACMG, American College of Medical Genetics and Genomics; AS, typical ultrastructural changes in the glomerular basement membrane of Alport syndrome; DC, disease causing; D, damage; EM, electron microscopic; ESKD, end-stage kidney disease; f, father; GBM, glomerular basement membrane; het, heterozygote; hemi, hemizygote; HU, hematuria; LP, likely pathogenic; m, mother; MsPGN, mesangial proliferative glomerulonephritis; ND, not determined; P, pathogenic; PU, proteinuria; VUS, variants of uncertain significance.
The allele frequency of COL4A3 variant c.4793T>G was 0.000033 in the Exome Aggregation Consortium (ExAC) and 5.61266E-05 in the Genome Aggregation Database (gnomAD), whereas not found in 1000 Genomes Project (1000G). The remaining 11 variants had not been reported in 1000G, ExAC, and gnomAD.
The variants with the Splice AI Δscore >0.2 were predicted to disrupt splicing.
The reference sequences are *COL4A3* (NM_000091.4), *COL4A4* (NM_000092.4) and *COL4A5* (NM_033380.2).

Table 2. Impact on abnormal splicing of the 9 unclassified variants in this study

No.	Gene	Location	DNA variant	Urine mRNA change	Expected change	Effect on protein
VUS-1	COL4A3	intron 37	c.3211-30G > T	r.3210_r.3211ins [3211-19_3211-1]	frame shift (insertion of 19 bp)	p. Gly1071Valfs*6
VUS-2	COL4A3	intron 14	c.828+5G>A	r.766_r.828del	Exon 14 skipping (63 bp)	p.256Asp_276Serdel21
VUS-3	COL4A4	intron 37-exon 38	c.3506-13_3528del	r. 3506_3577del	Exon 38 skipping (72 bp)	p.1170Pro_1193Glydel24
VUS-4	COL4A5	intron 48	c.4316-20T>A	r.4315_4316ins [4316-18_4316-1]	in-frame (insertion of 18 bp)	p. Pro1438_Gly1439ins6
VUS-5	COL4A5	intron 18	c.1033-10G>A	r.1032_r.1033ins [1033-8_1033-1]	frame shift (insertion of 8 bp)	p. Val345*
VUS-6	COL4A5	Exon 8	c.451A>G	r.439_r.465del	Exon 8 skipping (27 bp)	p. Pro148_Gly156del9
VUS-7	COL4A5	intron 18	c.1033-10_1033-2del	r.1033_1043del	frame shift (deletion of 11 bp)	p. Val345Thrfs*62
VUS-8	COL4A5	intron 26	c.2042-9 T>G	r.2042_2146del	Exon 27 skipping (105 bp)	p. Asp682_Gly716del35
VUS-9	COL4A5	Exon 32	c.2689 G>C	r. 2678_2767del	Exon 32 skipping (90 bp)	p. Thr894_Gly923del30

VUS, variants of uncertain significance

the probands or their family members were available in 9 of the 11 patients. Therefore, the 9 unrelated patients were enrolled in this study. Of them, 5 were diagnosed as AS (patient number 1, 4, 5, 7, and 9) and 4 were suspected with AS (patient number 2, 3, 6, and 8) according to the clinical information (Table 1). The median age at onset of the disease was 5 years (range 1–33 years). The rates of hematuria and proteinuria were 100% and 66.7%, respectively. Only 1 patient presented with abnormal serum creatinine. Six of them (6/9, 67%) had positive family history of hematuria or ESKD. Of 2 patients (patient number 7, 8) who underwent pure tone audiometry testing and ophthalmologic examination, none of them had hearing loss or ocular lesions.

Gene Variants at DNA Level

In total, 6 VUSs in *COL4A5*, 2 VUSs and 2 pathogenic variants in *COL4A3*, as well as 1 VUS and 1 likely pathogenic variant in *COL4A4* identified by WES were collected and confirmed by Sanger sequencing in the patients and their family members (Table 1). Eleven variants (11/12, 92%) were novel. Of the 9 VUSs, 7 were in the intronic areas of *COL4A3* to *COL4A5* genes and 2 were non-Glycine missense in *COL4A5* gene. Of the 9 patients, 3 would be genetically diagnosed as ARAS and 6 would be XLAS if the VUSs could be pathogenic.

Urine mRNA Analyses

All the 9 VUSs in *COL4A3* to *COL4A5* genes resulted in abnormal splicing by urine mRNA analysis (Table 2). The 2 VUSs in noncanonical splice site of *COL4A3* caused out-of-frame insertion and in-frame deletion of skipping exon 14, respectively. The VUS of deletion of 36bp from intron 37 to exon 38 in *COL4A4* caused in-frame deletion of skipping exon 38 in the transcript. Of the 6 *COL4A5* VUSs, 3 caused in-frame deletion of exons, 1 caused in-frame insertion, and the other 2 caused out-of-frame insertion and deletion, respectively (Figure 1). Therefore, all the 9 VUSs in *COL4A3* to *COL4A5* genes were evaluated as pathogenic variants according to American College of Medical Genetics and Genomics criteria. The 9 patients in this study obtained genetic diagnosis of AS, including 3 ARAS and 6 XLAS.

DISCUSSION

In this study, by analyzing urine *COL4A3* to *COL4A5* mRNAs, we proved the pathogenicity of 9 VUSs in these genes identified by WES. Furthermore, the reliability of spliceAI was only 44% (4 out of 9 proven splicing variants, Table 1) in this study, which indicated that many noncanonical splice site variants were likely to be missed. The present study underlies the usefulness of urine mRNA analysis for *COL4A3* to *COL4A5* in genetic diagnosis of AS.

In the recent decade, there is no doubt that genetic diagnosis of AS is greatly speeded up by NGS.[30] Nevertheless, the limitations and challenges raised by NGS are recognized by physicians, especially in patients with clinical diagnosis or highly suspected of AS. Some of them have complete negative results and some of them have VUSs in *COL4A3* to *COL4A5* genes detected by NGS. For these patients, to make a conclusive genetic diagnosis, it is necessary to analyze *COL4A3* to *COL4A5* genes further to identify the pathogenic variants or to do experiments to prove the pathogenicity of the identified VUSs. In our previous study, 6 deep intronic pathogenic variants leading to abnormal splicing were identified in 4 unrelated patients with Alport with negative NGS results by analyzing *COL4A3* to *COL4A5* mRNAs from urine or skin fibroblasts.[27] We demonstrated that urine *COL4A3* to *COL4A5* mRNAs analysis was a reliable and feasible method to detect splicing variants and to make up for the weaknesses of NGS in patients with Alport.

In the present study, 9 VUSs in Alport genes detected by NGS were proved to be pathogenic by analyzing urine *COL4A3* to *COL4A5* mRNAs of the patients. Of the 9 VUSs, 5 were in noncanonical splice site of Alport genes, 2 were small deletions from intron to exon, and the other 2 were non-Glycine substitutions. It indicates that various kinds of VUSs

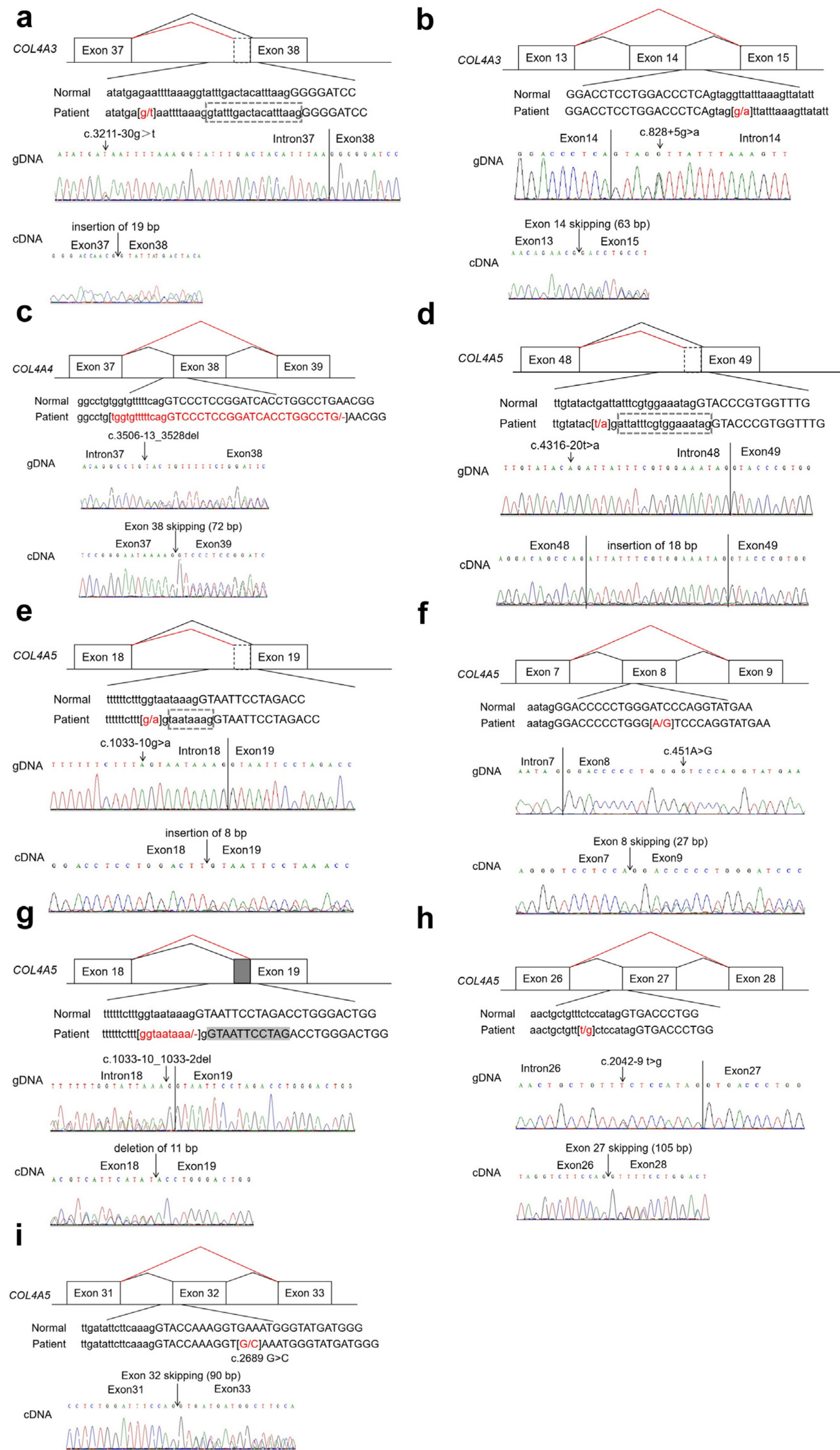

Figure 1. Nine variants of uncertain significance (VUS) and their consequences. The upper panels show schematics of aberrant splicing (red lines). Normal splicing is indicated by black lines. The original sequences are shown below and the 9 variants in patients' sequences are in red. Inserted sequences on transcripts are indicated by dotted box. Deleted sequences on transcripts are indicated by gray background. Flanking genomic DNA and cDNA sequences of either patients or the mothers are shown in lower panels. (a) VUS-1. c.3211-30G > T in intron 37 of COL4A3 produced an insertion of 19-bp in abnormal transcript in patient 1. (b) VUS-2. c.828+5G>A in intron 14 of COL4A3 resulted in exon 14 skipping, which created a transcript with a 63-bp deletion in patient 2. (c) VUS-3. c.3506-13_3528del (deletion 36bp) in the intron 37-exon 38 boundary of COL4A4 resulted in exon 38 skipping, which created a transcript with a 72-bp deletion in patient 3. (d) VUS-4. c.4316-20T>A in intron 48 of COL4A5 produced an insertion of 18-bp in abnormal transcript in patient 4. (e) VUS-5. c.1033-10G>A in intron 18 of COL4A5 produced an insertion of 8-bp in abnormal transcript in patient 5 and his mother. (f) VUS-6. c.451A>G in exon 8 of COL4A5 resulted in exon 8 skipping, which created a transcript with a 27-bp deletion in patient 6. (g) VUS-7. c.1033-10_1033-2delGGTAATAAA in intron 18 of COL4A5 created a transcript with an 11-bp deletion in patient 7. (h) VUS-8. c.2042-9 T>G in intron 26 of COL4A5 resulted in exon 27 skipping, which created a transcript with a 105-bp deletion in the mother of patient 8. (i) VUS-9. c.2689 G>C in exon 32 resulted in exon 32 skipping, which created a transcript with a 90-bp deletion in the mother of patient 9 (only urine sample available).

in Alport genes could result in abnormal transcripts. In our previous study, we found that about 56% splicing mutations were caused by variants at atypical or cryptic splice sites.[26] For example, the VUS in patient 9 was c.2689G>C in exon 32 of *COL4A5* and the effect on protein was initially predicted as a non-Glycine substitution (p. [Glu897Gln]). However, after analysis of *COL4A5* mRNAs, it was proved to cause exon 32 skipping (90 bp) in the transcript, suggesting it was a pathogenic splicing variant. In addition, we reported another pathogenic variant in deep intronic area c.2677+646 C>T in intron 31 of *COL4A5*, which also caused exon 32 skipping in the transcript.[27] Other pathogenic variants resulting in exon 32 skipping in *COL4A5* transcript was reported, including c.2678–1G>A in intron 31 of *COL4A5*,[31] and the last nucleotide substitution in exon 32 of *COL4A5*(c.2767G>C, p. [Gly923Arg]).[24] Therefore, various nucleotide changes in either introns or exons can result in the same effect on *COL4A5* transcript. The percentage of pathogenic variants causing abnormal splicing in *COL4A3* to *COL4A5* is likely more than reported. However, though SpliceAI was shown to achieve accuracy >90% for identifying splice-altering variants,[32] our data demonstrated this algorithm only identified 4 out of 9 proven splicing variants (44.4%, Table 1), indicating low sensitivity of *in silico* splice prediction methods.[22,27] Analyzing urine *COL4A3* to *COL4A5* mRNAs is an effective choice as the first step to determine the impact of VUS detected by NGS in patients who were diagnosed or suspected with AS.

To date, several retrospective studies have described strong genotype-phenotype correlations in patients with ARAS and males with XLAS. In our previous study of 101 patients with ARAS from Europe and China, we found that genotype in ARAS correlates with phenotype and response to therapy in favor of missense variants.[15] In males with XLAS, truncating variants in *COL4A5* were associated with "severe" disease with earlier onset kidney failure, and hearing loss and ocular abnormalities, which were reported in previous studies from European, Japanese, and Chinese populations.[8,9,33] In addition, it was reported that male patients with *COL4A5* splicing variants leading to in-frame transcripts had less severe phenotypes than those with out-of-frame transcripts.[31] Therefore, among the 3 patients with ARAS in this study, patient 1 was predicted to have an earlier onset age of ESKD because of 2 truncating variants in *COL4A3*, compared with the other 2 patients who had at least 1 nontruncating variant in *COL4A3* or *COL4A4*. Of the 4 males with XLAS in this study, patient 5 had out-of-frame transcript of *COL4A5* and the other 3 (patient 4, 8, and 9) had in-frame splicing transcripts. The results of our study provided information for genetic counseling and helped to predict the kidney prognosis for the patients and their family members.

Diffuse thinning of the GBM was considered as the earliest ultrastructural change of AS.[34] In our study, electron microscopy revealed that the width of the GBM was normal in patient 2 who underwent renal biopsy at age 3.7 years, which was in keeping with 2 published cases.[34,35] Vischini *et al*.[34] reported that in a male patient who manifested with hematuria and mild proteinuria at age 3 years, electron microscopy demonstrated normal GBM appearance and thickness in the first renal biopsy performed at age 9 years and diffuse GBM thinning without basket-weaving and lamellation in the second renal biopsy performed at age 14 years, whereas negative a3(IV) and a5(IV) chains immunostaining in the GBM and normal expression of α5 (IV) in Bowman's capsule were observed in twice biopsies. Storey *et al*.[35] had a female patient with ARAS and COL4A3 heterozygous pathogenic variants c.713del (p. [Pro238Argfs*9]) and c.1918G>A (p. [Gly640Arg]), showing normal GBM by electron microscopy when she underwent kidney biopsy at age 6 years. These findings highlight that genetic testing and collagen IV immunostaining are especially helpful for making correct diagnosis in children, who were suspected of AS and had normal GBM.

The limitation of this study is the small number of patients and the young median age preventing us from further genotype-phenotype correlation analysis. Long-term follow-up of these patients is necessary in the future.

In summary, we reported 9 novel *COL4A3* to *COL4A5* variants that result in abnormal splicing, which provided further evidence for abnormal splicing caused by noncanonical splice site variants and substitutions in exons of Alport genes. Furthermore, we suggest routine use of urine *COL4A3* to *COL4A5* mRNAs analysis to determine the pathogenicity of VUSs identified by NGS in patients with clinical diagnosis or suspicion of AS.

DISCLOSURE

All the authors declared no competing interests.

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DATA AVAILABILITY

The data were stored in the online registry of pediatric hereditary kidney diseases in China.

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