## Original Article Determination of the pathogenicity of a novel COL4A5 missense variant by CRISPR-Cas9 in kidney podocytes

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Abstract: Objective: The main purpose of this research was to investigate the influence of the novel COL4A5 missense mutation on collagen type IV. Methods: Clinical data and detailed family history were collected. Targeted nextgeneration sequencing (NGS) was applied to examine potential pathogenic variants in COL4A3, COL4A4, COL4A5 genes in the proband, and then the variants were analyzed using bioinformatics tools and pedigree analysis. The CRISPR/Cas9 gene editing was used to knock in potential pathogenic variants in human podocytes, and then western blot analyses and immunofluorescence assays were used to measure COL4A5 protein expression. Results: Three patients (I: 2, II: 1 and II: 2) presented with microscopic hematuria and proteinuria, and the patient II: 1 progressed to abnormal renal function by age 14. A novel missense variant, c.2641G>A (p. Gly881Arg), located in exon 31 of COL4A5 gene, was chosen as a possible pathogenic variant. The variant significantly decreased collagen IV  $\alpha$ 5 chain expression in CRISPR/Cas9 gene edited podocytes. Conclusion: By conducting NGS and CRISPR/Cas9 geneediting of podocytes, a novel COL4A5 missense variant, c.2641G>A (p. Gly881Arg), was confirmed to be the genetic defect of X-linked Alport syndrome in the Chinese family. Our findings extend the genetic spectrum of X-linked Alport syndrome with COL4A5 mutations and provide a method for evaluating the functional significance of novel COL4A5 missense variants in vitro.

Keywords: COL4A5 gene, CRISPR/Cas9, X-linked Alport syndrome, podocyte

#### Introduction

Alport syndrome (AS) is a rare type IV collagen hereditary kidney disease which is caused by COL4A3, COL4A4, and COL4A5 gene mutations. The spectrums of phenotype range from microscopic hematuria with a relatively slow progression to progressive renal function loss and end-stage renal disease (ESRD) with other abnormalities like bilateral sensorineural hvpoacusis and ophthalmic complications. The prevalence of AS is 1/5000-1/10000. Approximately 80-85% of AS cases were X-linked inheritance pattern and variants in COL4A5, which encodes the collagen type IV  $\alpha$ 5 chain [1]. The patients with COL4A5 mutation have symptoms from asymptomatic microscopic hematuria and persistent proteinuria to ESRD. However, Male patients with hemizygous COL4A5 mutations are more severely affected, and usually develop to ESRD before the age of 30 years [2-4].

As far as it is known, more than 1168 different COL4A5 mutations have been reported, and approximately 45% of pathogenic mutations are missense mutations [1, 5]. The pathogenic COL4A5 mutations could interfere with the normal folding of collagen  $\alpha 5$  triple helices and result in the complete or partial absence of collagen type IV  $\alpha 5$  in mature glomerular basement membranes (GBM) [6]. Now, it is found that many COL4A5 gene variants could lead to XLAS, and the pathogenicity of the novel variants has been determined by basic population information, computational and predictive analysis, functional assessment, segregation data analysis, and others. According to the specific standard terminology, the missense variants were mostly described as likely pathogenic with uncertain significance because of absent of functional evidence. There were few researches on functional identification in vitro about COL4A5 variant.

Therefore, this article reports the study of a novel *COL4A5* missense mutation in a Han-Chinese family with renal disorder and the *COL4A5* function identification through CRI-SPR/Cas9-mediated genomic editing in kidney podocytes.

#### Materials and methods

#### Ethics statement and clinical evaluation

The research was authorized by the Ethics Committee of the Shanghai Children's Hospital (No. 2020R084-F01). All patients provided written informed consent. A 2-generation, 4-individual Chinese family was enrolled at Shanghai Children's Hospital, China (Figure 1A). Associated clinical and phenotype information, including clinical symptoms, urine test, renal function assessment, ocular fundus examination, slit-lamp biomicroscopy, and hearing examinations were collected. The proband underwent kidney biopsy, and examined with light microscopy, immunofluorescence, and electron microscopy (II: 1). Genomic DNA of all family members were extracted from blood with the GentraPuregene Kit (Oiagen, Germany) and used for sequencing (I: 1, I: 2, II: 1, II: 2).

#### Diagnostic NGS and sanger sequencing

Targeted NGS was performed to the proband to examine pathogenic variants and gene modifications in COL4A3, COL4A4, and COL4A5 genes which are responsible for the renal disease phenotype. The sequencing was performed by Huada Clinical Inspection Center, Shenzhen, China. SureSelectXT based target enrichment system was used to capture targeted gene. Gene sequence was performed by Illumina paired-end multiplexed sequencing. Variants were analyzed with bioinformatics tools and classified based on the classification of the American College of Medical Genetics (ACMG) [7]. For further identification, the candidate variants in 4 pedigree individuals were analyzed using sanger sequence. The primers (5'-ACTTGATGTTCCAGGACCCC-3' and 5'-TGGG-AATTATCTACCAGAGTCGT-3') were used for PCR amplification and sanger sequencing.

#### Cell cultures

Conditionally immortalized human podocyte cell lines (AB8/13, University of Bristol, UK)

were routinely cultured in the RPMI 1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA). Podocytes were proliferated under permissive conditions at 33°C and supplemented with 1% Insulin-Transferrin-Selenium (Life Technology, USA). After being cultured to approximately 60% confluency, podocytes were sub-cultured in an incubator at 37°C under 5% CO<sub>2</sub> for full differentiation. After 10-14 days differentiation, the fully differentiated podocytes were generated and maintained for future experiments.

#### CRISPR-Cas9 gene editing of podocytes

(1) Design and construction of guide RNAs (gRNA) and repair template. The gRNA was designed using online software (crispr.mit.edu). The target DNA sequence (COL4A5 exon 31 sequence) was submitted for choosing optimal gRNA (20 nucleotides followed by the NGG PAM). An oligonucleotide containing the gRNA and BsmBI compatible 5' overhangs was synthesized: forward sequence 5'-CACCgTAGGA-CCTCCAGGATCACCA-3' and reverse sequence 5'-aaacTGGTGATCCTGGAGGTCCTAc-3' (Genechem, Shanghai). Optimal gRNA was then ligated into a U6-sgRNA-EF1a-Cas9-FLAG-P2A-EGFP (GV393, Genechem, Shanghai) expression system utilizing the BsmBI restriction site. The GV393 is an expression system containing a U6 promoter driving the sgRNA expression with anEF1a promoter driving the Cas9. Sanger sequencing was used to verify the GV393 sequence. Repair template for facilitating homology directed repair (HDR) was synthesized, which included a restriction enzyme site Mlul/HindIII (GV469, Genechem, Shanghai).

(2) Delivery. For delivery of gRNA and repair template into cells, podocytes were re-plated onto 6-well plates and cultured in RPMI 1640 medium supplemented with 10% FBS. According to the cell counting, multiplicity of infection (MOI) and virus titer, 20 ul GV393 vector, 20 ul GV469 vector and 2 ul PolyBrene were dropwise added to culture medium for transfection. Subsequently, the cells were cultured in incubator with 37°C, 5% CO<sub>2</sub> for 12-16 h, and then replaced the medium. After 96 h posttransfection, GFP expression was checked. Cells were re-suspended and prepared for FACS single-cell sorting. Corresponding control groups were treated with sgRNA-CON251 (Genechem, Shanghai).

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**Figure 1.** Clinical data of the AS family. A. Pedigree of the AS family. N: normal; V: the *COL4A5* c.2641G>A variant. Arrow indicates the proband. B. Sequence of hemizygous c.2641G>A variant (II: 1). C. Sequence of heterozygous c.2641G>A variant (II: 2). D. Renal pathological examination of the proband (II: 1). Original magnification: ×400; Scale bars: 50  $\mu$ m. E. Immunofluorescence image for collagen type IV  $\alpha$ 2 chains expression in renal of the proband (II: 1). Original magnification: ×200; Scale bars: 100  $\mu$ m. F. Immunofluorescence image for collagen type IV  $\alpha$ 5 chains expression in renal of the proband (II: 1). Original magnification: ×200; Scale bars: 100  $\mu$ m. G. Renal electron microscopy examination of the proband (II: 1). Original magnification: ×5000; Scale bars: 5  $\mu$ c.

(3) Confirmation of gene editing. Single-cell colonies were transferred and expanded in 10 cm culture dishes. DNA was purified by Genomic DNA Extraction Kit (Qiagen, Germany) for PCR amplification of target sequence. PCR products were detected by sanger sequencing to verify the homozygous c.2641G>A variant of *COL4A5* gene.

harboring COL4A5 c.2641G>A (p. Gly881Arg) variant (N: normal)			
Subject	I: 2	II: 1	II: 2
Sex	female	male	male
Genotype	Heterozygote	Hemizygote	Hemizygote
Age (years)	42	18	9
Renal function (ml/min)	eGFR>90	eGFR: 30-60	eGFR>90

Yes

No

Yes

Ν

Ν

Yes

Yes

Yes

Ν

Ν

 Table 1. Clinical and genetic information of 3 members

#### Western blotting

Microscopic hematuria

Audiological examination

Ophthalmic examination

Gross hematuria

Proteinuria

Total proteins of podocytes were extracted using RIPA buffer (Beyotime, China). The BCA assay (Pierce, USA) was used to determine the concentrations of proteins. According to the protein concentrations, equal amounts of podocytes total proteins (30 µg/well) were denatured and subjected to 10% SDS-PAGE. The proteins were then transferred to PVDF membranes (Bio-Rad, USA). After blocking in TBST supplemented with 5% skimmed milk, the membranes were incubated with the primary antibody overnight at 4°C (Rabbit anti-human COL4A5, 1:500, Santa cruze, USA). Sequentially, goat anti-rabbit IgG-HRP linked antibody (1:10000, Jackson, UK) was incubated for 2 h at room temperature. Finally, the immunoreactive bands were visualized using chemiluminescence substrate (Perkin Elmer, USA). And the images were captured using LAS4000IR (Fujifilm, Japan). The Image Reader LAS-4000 software was used to analyze the proteins expression.

#### Immunofluorescence analysis

Podocytes were fixed with 4% para-formaldehyde (Sigma) for 30 min at room temperature, and then washed by PBS for three times. Subsequently, podocytes were blocked in 2% BSA for 30 min. After PBS washing for three times, podocytes were stained with primary antibody (COL4A5, diluted at 1:500, 37°C for 1 h). The secondary antibody conjugated with Alexa Flour 488 (Abcam, UK) was incubated at a dilution of 1:1000 for 1 h at room temperature and washed respectively. The nucleus was counter stained using Fluoroshield mounting medium with DAPI (1:10000, Invitrogen, USA). Fluorescent images were captured by the confocal microscopy (Leica, Germany) and were analyzed using Image J.

#### Statistical analysis

Data were expressed as mean ± standard deviation (SD), and the SPSS version 19.0 was used for statistical analysis. Intergroup comparisons were tested by one way analysis of variance (ANOVA), followed by

the least-significant difference test. P-Values less than 0.05 was considered statistically significant.

#### Results

Yes

No

Yes

Ν

Ν

#### Clinical findings

Clinical characteristics and genetic information of family members who were harboring COL4A5 novel variant are presented in **Table 1**. All three patients (I: 2, II: 1 and II: 2) presented microscopic hematuria and persistent proteinuria, while the proband (II: 1) showed gross hematuria after a respiratory tract infection and abnormal kidney functions by age 14 (Figure 1A). No other AS related symptoms (sensorineural hearing loss, ophthalmological change) were found in any family members. The proband (II: 1) underwent a renal biopsy. Light microscopy showed mild mesangial proliferative nephropathy (MsPGN), foam cells in renal interstitial, segmental thickening, and light staining change in the GBM (Figure 1D). Immunofluorescence showed normal expression of collagen type IV  $\alpha$ 2 chains in the GBM, and the  $\alpha$ 5 chains expression was negative (Figure 1E, 1F). Electron microscopy showed mild MsPGN, with no apparent GBM abnormalities (Figure 1G).

#### Variant screening

Targeted NGS was performed on the proband (II: 1), which discovered a novel missense variant c.2641G>A (p. Gly881Arg) in exon 31 of COL4A5 (NM\_000495) (Figure 1B). The prediction of protein function was "damaging" by SIFT and PolyPhen-2. Related family members were

identified by Sanger method, and the mother of the proband (l: 2) had the c.2641G>A heterozygous variant (**Figure 1C**), the younger brother of the proband (ll: 2) had the same hemizygous variant, while his father (l: 1) was wild type. The frequency of c.2641G>A in normal population is extremely low, which has not been reported. No suspected pathogenic variant in the COL4A3/4 genes was found in the proband. Based on these findings, only the c.2641G>A (p. Gly881Arg) variant in *COL4A5* was considered as a possible pathogenic variant for further analysis and validation.

# CRISPR-Cas9 gene editing and collagen $\alpha 5$ expression in podocytes

Three groups of cells (COL4A5-edited group, editing control group, blank control group) were stably transfected with corresponding vector. After extraction of total proteins of podocytes, western blot analysis was used to detect collagen  $\alpha$ 5 protein expression. The protein expression of collagen  $\alpha$ 5 in COL4A5-edited group was significantly reduced than that of the editing control group and the blank control group (*P* < 0.01) (**Figure 2B**).

Furthermore, we used immunofluorescence to measure the location and level of collagen type IV  $\alpha$ 5 expression in different groups. The  $\alpha$ 5 chain was mainly expressed in cytoplasm. The fluorescence intensity of collagen type IV  $\alpha$ 5 in COL4A5-edited group was significantly reduced than that in the editing control group and the blank control group (**Figure 2A**). The novel variant in *COL4A5* gene significantly reduced  $\alpha$ 5 chain expression in podocytes.

#### Discussion

XLAS is the major form of AS which is caused by mutations in the *COL4A5*. Male cases mostly present severe phenotypes [8], and 90% of patients progress to ESRD before age 40 [9]. However, the probability of developing ESRD of the heterozygous females was 12%, and the phenotypes were widely varied from normal urine to ESRD [9, 10]. Now the phenotypic variability in females was considered to be associated with X-chromosome inactivation skewing and allelic heterogeneity.

The diagnosis of XLAS is complicated, which is based on the renal biopsy, clinical symptoms,

extrarenal manifestations, and family history. However, the family history of approximately 10-15% XLAS cases was negative; those patients were harboring COL4A5 de novo mutations [11-13]. For definitive diagnosis of AS. particularly in isolated hematuria children with negative family history, genetic testing is becoming a gold standard of AS diagnosis. Genetic diagnosis can make early diagnosis of AS. Through preemptive therapy, we can slow the progression of AS to ESRD [14]. When variants are detected in COL4A5, it is essential to determine whether they are pathogenic. This part is always difficult, particularly when the variants have not been reported. Now, the influence of variants in protein function can be predicted by bioinformatics software, such as Polyphen or SIFT, but rarely confirmed by expression experiments. It is vital to perform protein expression analysis to confirm the pathogenicity of novel variants.

In this study, we described 3 patients (two males and one female) belonging to one AS family. The clinic symptoms of the proband were more severe than those of his mother, such as more progressive hematuria, proteinuria, and renal function damage. Renal biopsy was performed to the proband and the expression of α5 (IV) in GBM was negative. For definitive diagnosis, a screening of the COL4A3/4/5 gene was made for the proband and a hemizygote missense variant c.2641G>A (p. Gly-881Arg) in exon 31 of COL4A5 gene was found. Pedigreed analysis revealed that the patient's young brother had the same hemizygous variant, and his mother had the c.2641G>A heterozygous variant. To be certain that the observed developmental defect of  $\alpha 5$  (IV) was caused by the missense variant c.2641G>A, we additionally performed experiments using COL4A5 c.2641G>A point mutation podocytes by CRISPR/Cas9 editing. Compared to wild type, there was a significant reduction in collagen  $\alpha 5$ expression in podocytes transfected with CAS9, gRNA and repair template vectors which made the c.2641G>A variant cell model. This is the first report comprehensively exploring a COL4A5 novel missense variant assumed to be pathogenic by CRISPR/Cas9 editing.

Until now, more than 1,168 *COL4A5* gene mutations have been reported that are associated with XLAS. The missense mutation which

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**Figure 2.** CRISPR-Cas9 gene editing and collagen  $\alpha$ 5 expression in podocytes (n=5). A. The expression of collagen  $\alpha$ 5 in editing control group and blank control group was mainly in cytoplasm, and the expression of collagen  $\alpha$ 5 in COL4A5-edited group was significantly lower than that of editing control group and blank control group. Original magnification: ×630; Scale bars: 25 µm. B. The protein expression of collagen  $\alpha$ 5 in COL4A5-edited group was significantly reduced than that of editing control group and blank control group was significantly reduced than that of editing control group and blank control group was significantly reduced than that of editing control group and blank control group.

accounted for 45% of all AS mutations is the most common mutation type of *COL4A5* gene [1, 5]. Different from patients with insertion

and deletion variants, which cause the reading frame shift of the *COL4A5* gene, the phenotype of missense mutation patients varies greatly.

Fifty percent of patients with missense mutations progress to ESRD before age 30. But in patients with missense mutations, hearing loss and ESRD were observed after 50 years old [8, 15]. Because of absent of functional evidence, the missense variants of the COL4A5 gene were mostly described as likely pathogenic with uncertain significance. Therefore, the functional evidence is needed to confirm whether the missense variant of the COL4A5 can cause abnormal  $\alpha 5$  (IV) expression and consequently cause AS. Through the AS podocyte model constructed by CRISPR/Cas9 editing, our research confirmed the effectiveness of COL4A5 missense variant efficiently, and determined the pathogenicity of the novel gene.

The most perfect approach to predict pathogenicity of novel variant is to perform functional study using the patients' affected organ or tissues. But it is always difficult to obtain renal tissues to identify tissue-specific gene expression. Besides renal tissue, peripheral blood lymphocytes, urine-derived cells, and skin biopsies from patients also have been studied as evidences for the diagnosis of AS [16-23]. It may be workable to confirm the pathogenicity of a novel variant by transcriptional analysis of mRNA isolated from different kind of samples. But this method is also difficult because the transcripts are unstable within tissues [24]. It has been reported that detecting the expression level of  $\alpha 5$  chain in the hair root is effective, but the sensitivity and specificity have not been fully determined [18]. Thus, developing another method to confirm the pathogenicity of uncertain significant novel variant is urgently needed.

The CRISPR/Cas9 technology can be used to edit specific DNA sequences within genomes rapidly and efficiently. Herein, we adopted CRISPR/Cas9-mediated genomic editing to specifically edit a genomic region in human podocytes. We revealed that CRISPR/Cas9mediated genomic editing can be used to knock in specific sequences (e.g., novel gene variant) into target genomic locus *via* homology directed repair. Our findings demonstrated the application of CRISPR/Cas9-mediated genomic editing in human podocytes gene editing, which can be widely used for functional validation researches of novel *COL4A5* variants. These gene-edited podocytes can determine the pathogenicity of the novel gene, and provide new insight into the cellular mechanisms of AS. In summary, the method we described herein provides an efficient and relatively rapid method for confirming the pathogenicity of novel *COL4A5* missense variants *in vitro*.

### Conclusions

By conducting NGS and CRISPR/Cas9 geneediting of podocytes, a novel *COL4A5* missense variant, c.2641G>A (p. Gly881Arg), was confirmed to be the genetic defect of XLAS in this Chinese family. Our findings extend the genetic spectrum of XLAS with *COL4A5* mutations and provide a method for evaluating the functional significance of novel *COL4A5* missense variants *in vitro*.

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#### Disclosure of conflict of interest

#### None.

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