Kidney Medicine

Severe Clinical Phenotype in Alport Syndrome Due to 2 *COL4A4* Exon-Skipping Events

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Alport syndrome (AS) is a genetically heterogeneous disorder caused by mutations in *COL4A3*, *COL4A4*, or *COL4A5*, leading to progressive kidney dysfunction. Although genetic screening has advanced, many cases remain undiagnosed due to deep intronic splice-site variants. We report a male patient diagnosed with autosomal recessive AS, characterized by hematuria, proteinuria, and chronic kidney disease progression. Initial kidney biopsy at age 10 revealed glomerular basement membrane thinning and focal segmental glomerulosclerosis, whereas targeted deoxyribonucleic acid sequencing failed to detect pathogenic variants. Over 15 years, kidney function declined, and a second biopsy showed severe glomerular basement membrane abnormalities with multilamellated structures. Whole-transcriptome sequencing revealed 2 events of exon skipping, specifically at exons 27 and 38 of the *COL4A4* gene, which were verified by exon-specific PCR and Sanger sequencing. Intronic regions analysis revealed 2 heterozygous variants positioned 78 bp downstream of exon 27 and 8 bp upstream of exon 38, though their role in aberrant splicing remains uncertain. Immunofluorescence analysis confirmed disrupted α3α4α5(IV) heterotrimer assembly. This is the first documented case of dual exon-skipping events in *COL4A4*, highlighting their contribution to disease severity. Our findings emphasize the need for ribonucleic acid–based diagnostics and raise questions about potential benefit of exon-skipping therapy in autosomal recessive AS.

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Alport syndrome (AS) was first described by Arthur Cecil Alport in 1927.[1] In 1990, *COL4A5* was identified as the causative gene for X-linked AS, followed by the discovery of *COL4A3* and *COL4A4* in 1994 as the genetic basis for autosomal dominant and recessive AS.[2] These genes encode key components of type IV collagen, which is essential for maintaining glomerular basement membrane (GBM) integrity beyond infancy.[3,4] Despite nearly 35 years of genetic research, current screening tests detect only about half of the pathogenic variants in patients with hematuria or thinned GBM.[4–6] Deep intronic variants, leading to exon skipping at the transcript level, may account for a significant proportion of undiagnosed cases.[7] Clinically, AS exhibits significant heterogeneity, ranging from isolated hematuria to progressive glomerular dysfunction, often culminating in end-stage renal disease in early adulthood, particularly in males with X-linked AS and individuals with autosomal recessive AS.[8] Although extrarenal manifestations such as sensorineural hearing loss and anterior lenticonus are primarily associated with X-linked and autosomal recessive AS, they further contribute to the disease burden.[9] Recent advancements in personalized medicine have suggested different approaches to restore the α3α4α5(IV) structure, including exon-skipping therapy.[10–12] However, genotype–phenotype correlations, particularly concerning exon-skipping events, remain poorly understood. Understanding their phenotypic consequences is crucial for developing targeted therapies, as these cases provide models for predicting treatment outcomes.

CASE REPORT

In a male patient who had undergone a hemi-nephroureterectomy and ureterocele excision before the age of 1 year, proteinuria developed at the age of 10 years, along with persistent hematuria. At that time, he weighed 40 kg, was 157 cm tall, and had a normal blood pressure of 122/62 mm Hg. His blood urea level was within the upper limit of normal (urea: 7.3 mmol/L), whereas his kidney function, based on estimated glomerular filtration rate, remained normal (creatinine: 61 μmol/L, estimated glomerular filtration rate: 126 mL/min/1.73 m^2). He had hematuria, with brown-colored urine in the morning, and proteinuria reached 1.5 g per day. Consequently, he was prescribed 10 mg of the angiotensin converting enzyme inhibitor, fosinopril, daily. The patient did not exhibit typical ocular abnormalities or sensorineural hearing loss. A kidney biopsy obtained at that time showed 21 glomeruli, with 3 global and 2 segmental glomerulosclerosis. Interstitial fibrosis and tubular atrophy was 10%. Immunofluorescence staining of α3 and α5 collagen IV showed that GBM was negative for α5 but positive in Bowman's capsule and tubular basement membrane (2+). α3 staining was segmentally and mildly positive in GBM (+) but negative in Bowman's capsule and tubular basement membrane, whereas α1 staining was positive in GBM (+) and strongly positive in tubular basement membrane (3+) (Fig 1C and D). Electron microscopy investigation showed diffuse GBM thinning, with 10% to 15% thickened and multilamellated in a "basket-weave" pattern,

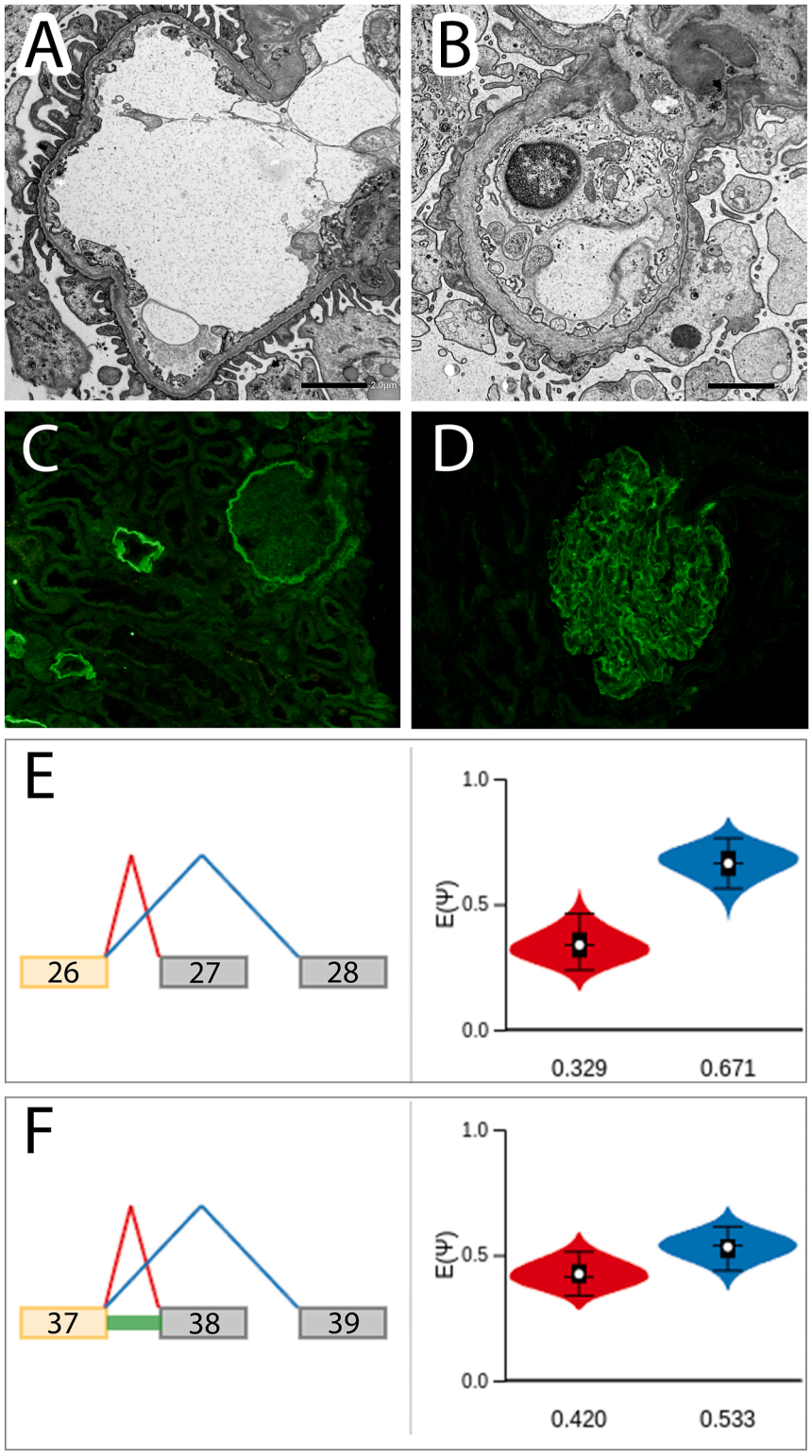

Figure 1. Ultrastructural, immunofluorescence, and splicing analysis of the *COL4A4* gene in kidney samples. (A) Diffuse thinning of the GBM and normal podocyte foot processes in the first biopsy. (B) Thickening of the GBM, with a "basket-weave" pattern and diffuse effacement of podocyte foot processes in the second biopsy. (C) Positive immunofluorescence for α5 collagen IV in Bowman's capsule and TBM but negative in GBM. (D) Segmentally and mildly positive immunofluorescence for α3 collagen IV in the GBM but negative in Bowman's capsule and TBM. (E, F) Exon-skipping event of exons 27 and 38. The left panels depict alternative splicing events with different exon inclusion levels (red as retaining and blue as skipping event). The right panels show violin plots representing splicing event distribution (red as retaining and blue as skipping event). TBM, tubular basement membrane.

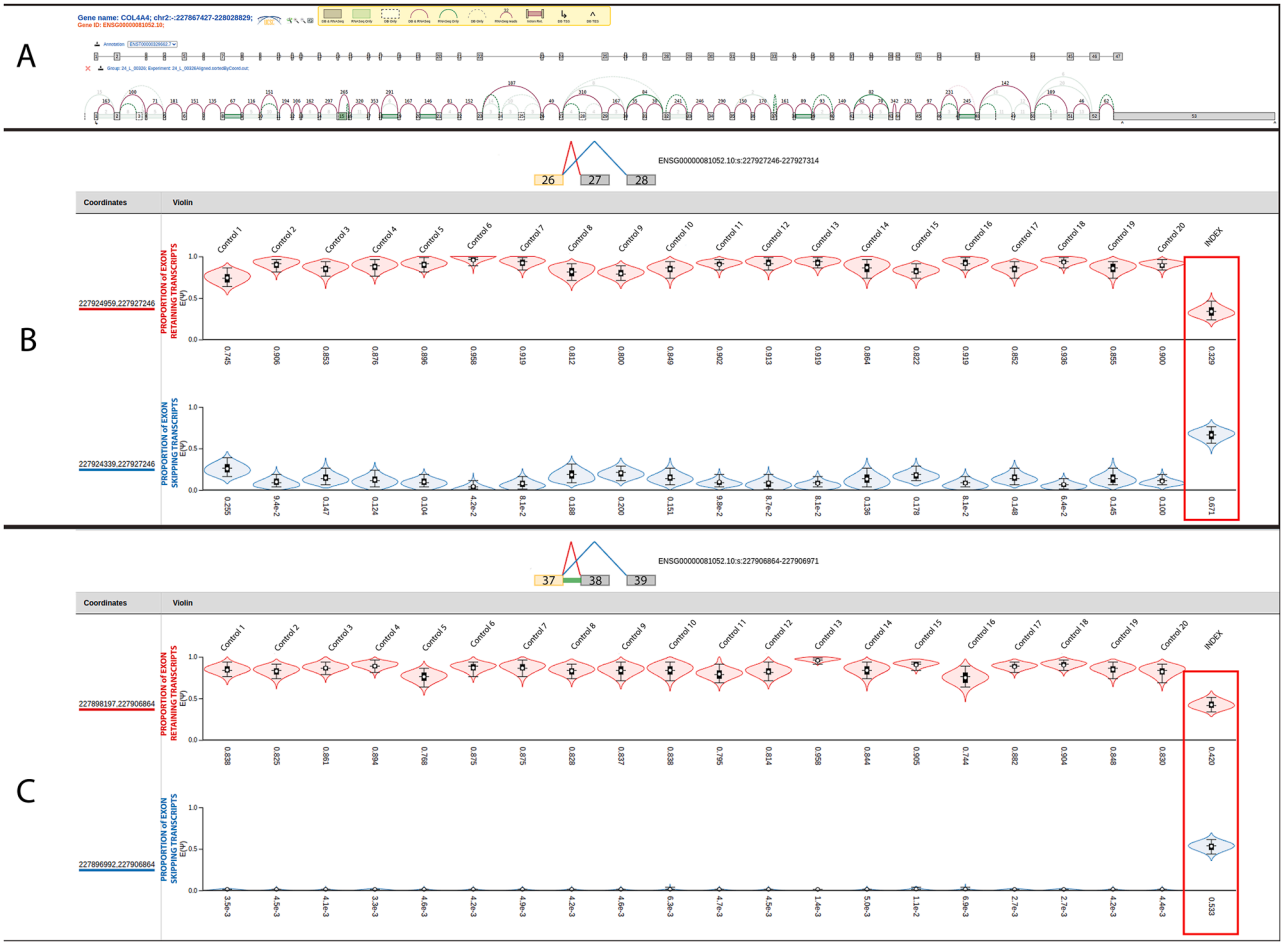

Figure 2. Visualization of alternative splicing events in the *COL4A4* gene. (A) Spliceograph with annotated versus de novo exons, junctions, and retained introns, including observed raw read coverage for each intron or junction. (B) Violin plot visualization of local splicing variations at exon 26–27 and 26–28 junctions. The red (exon retaining) and blue (exon skipping) violin plots depict alternative splicing event distributions, with black dots indicating mean expression levels across samples. (C) Violin plot representation of local splicing variations at exon 37–39 junctions, following the same color-coding scheme. The boxed region indicates an alternative splicing event with a shift in expression levels in index sample.

without podocyte foot process effacement (Fig 1A). Together, these investigations during the first biopsy suggested autosomal recessive AS with a mutation in the α3 or α4 collagen IV chain. However, targeted deoxyribonucleic acid sequencing of peripheral blood obtained at the time of the first biopsy did not identify any pathogenic variants in the exonic or canonical splice-site regions of the *COL4A3*, *COL4A4*, and *COL4A5* genes.

During subsequent regular follow-up, his chronic kidney disease progressively worsened. At the age of 25 years, when he weighed 85 kg and was 188 cm tall, his blood pressure remained normal at 119/68 mm Hg. His kidney function gradually declined (urea: 11.1 mmol/L, creatinine: 141 μmol/L, estimated glomerular filtration rate: 59 mL/min/1.73 m^2). Over the years, his proteinuria increased to over 3 g per day, prompting an increase in antiproteinuric therapy. By the time of the second biopsy at the age of 25 years, with a fosinopril dose of 40 mg per day, proteinuria did not exceed 2 g per day. Throughout this period, he underwent regular ophthalmologic and audiometric examinations every 2 years, with no development of ocular changes or hearing impairment. The second biopsy showed 16 glomeruli, with 6 global and 1 segmental glomerulosclerosis. The interstitial fibrosis and tubular atrophy increased to 30%. Electron microscopy revealed thicker capillary loops when compared to first biopsy, irregular GBM thickening with splitting and fragmentation, and diffuse podocyte foot process effacement (Fig 1B). Repeated targeted deoxyribonucleic acid sequencing during second biopsy, along with subsequent multiplex ligation-dependent probe amplification analysis, and whole exome sequencing on peripheral blood detected no pathogenic variants in the exonic or canonical splice-site regions of *COL4A3*, *COL4A4*, and *COL4A5*.

Whole-transcriptome sequencing of ribonucleic acid from the second kidney biopsy identified 2 significant

exon-skipping events in the *COL4A4* (NM_000092.5) transcript (Fig 1E and 1F). Modeling Alternative Junction Inclusion Quantification was used to detect and quantify alternative splicing events. Splice graphs were constructed, and percent spliced-in values were calculated for splicing analysis.[13] Both exon-skipping events were validated using exon-specific primer sets, followed by Sanger sequencing. To quantify the relative abundance of local splice variations, the percent spliced-in values were compared between the index patient and a cohort of 20 unrelated kidney biopsy samples, revealing significant differences in exon inclusion levels (Fig 2). Evaluation of intronic regions from targeted deoxyribonucleic acid sequencing identified a heterozygous single nucleotide polymorphism (SNP) (rs11898094) located 78 bp downstream of exon 27 and a heterozygous SNP (rs761588725) located 8 bp upstream of exon 38. Segregation analysis of the index patient's parents confirmed that SNP-rs11898094 was inherited from the father, whereas SNP-rs761588725 was inherited from the mother.

Based on the kidney histopathological findings and the results of whole-transcriptome analysis, the final diagnosis of autosomal recessive AS was established.

DISCUSSION

To the best of our knowledge, this is the first report of a patient with 2 exon-skipping events detected at the ribonucleic acid level in *COL4A4*, presenting with a severe clinical course of progressive nephropathy characteristic of AS. Both skipping events preserve the reading frame; however, due to the unavailability of parental ribonucleic acid samples, segregation analysis could not be performed, leaving the possibility of a de novo event. Additionally, because segregation analysis was not available, we could not determine the phase of the skipping exons. SNP analysis confirmed paternity and maternity, and both parents were asymptomatic, without any signs of hematuria or proteinuria.

Although in silico analysis initially predicted exon 27 skipping as benign,[4] recent NanoLuc-based collagen IV heterotrimer assays demonstrated that exon 27 skipping disrupts α3α4α5(IV) assembly and secretion.[14] Additionally, exon 27 skipping has been associated with hematuria and albuminuria, suggesting a likely pathogenic effect. In our study, analysis of intronic sequences flanking exon 27 identified SNP rs11898094, which is located 78 bp downstream. This SNP is common in the general population (gnomAD: 12.4%) and classified as benign in ClinVar (ID: 683328). SpliceAI predicted no effect on splicing. While rs11898094 has been reported in linkage disequilibrium with exon 27 skipping, in vitro studies suggest it is not the causal variant.[14] Notably, in this patient, the exon 27 skipping transcript appeared to be more abundant than the exon-retaining transcript. Several factors may contribute to this observation, including alterations in the splicing regulatory machinery or cis-acting elements that promote exon 27 skipping. The second exon-skipping event in our study, affecting exon 38 of *COL4A4*, has previously been linked to a 36 bp deletion spanning intron 37 to exon 38.[7] However, this deletion was not observed in our patient. Instead, the SNP rs761588725, located 8 bp upstream of exon 38, was identified. This SNP is rare in control populations (gnomAD: 0.000269%) and classified as a variant of uncertain significance or likely benign in ClinVar (ID: 447187). SpliceAI analysis predicted a low probability of splicing impact. In the literature, rs761588725 has been observed in a single family with benign familial hematuria,[15] but its role in exon 38 skipping remains unclear, necessitating further functional studies. Consequently, the precise molecular mechanisms underlying exon 27 and exon 38 skipping in this case remain undetermined.

Numerous studies have established a correlation between the type of pathogenic variant and disease severity in AS. Truncating variants in *COL4A5* are associated with severe, rapidly progressive renal decline, often accompanied by ocular abnormalities and hearing loss. By contrast, in-frame exon-skipping variants generally result in milder phenotypes.[16-19] These findings support the potential application of exon-skipping therapy as a therapeutic approach for patients with Alport carrying truncating or frameshift variants. However, in compound heterozygous autosomal recessive cases involving *COL4A3* or *COL4A4*, the feasibility of exon-skipping therapy as a mutation-correcting strategy remains uncertain because of the lack of genotype–phenotype correlation data. To our knowledge, no previously reported cases have described double exon-skipping events in any of the *COL4A* genes. In our case, a heterozygous in-frame deletion in 2 separate exons resulted in a severe autosomal recessive AS phenotype, underscoring the pathogenic impact of in-frame exon deletions.

In summary, our findings provide novel insights into the clinical impact of dual exon skipping and suggest that the loss of 2 exons results in a substantial deterioration of heterotrimeric collagen IV in GBM. This structural deficiency contributes to a severe AS phenotype, with a high probability of progression to end-stage renal disease.

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