Case Report Article | J Adv Med Biomed Res. 2025; 33(159): 341-347

Category: Clinical Medicine | Peer Reviewed | Open Access

Journal of Advances in Medical and Biomedical Research | ISSN:2676-6264

A Canonical Splice Site Variant in the Last Intron of the *COL4A5* Gene Causing X-Linked Alport Syndrome in an Iranian Family: A Case Report

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Article Info

10.30699/jambr.33.159.341

Received: 2025/04/14;
Accepted: 2025/09/05;
Published Online: 29 Sept 2025;

Use your device to scan and read the article online

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ABSTRACT

Alport syndrome (AS) is an inherited disorder of collagen type IV causing progressive renal disease, sensorineural hearing loss, and ocular abnormalities with variable severity. Here, we report a splice site variant in the *COL4A5* gene, leading to X-linked Alport syndrome (XLAS) in two siblings from a non-consanguineous Iranian family. The proband was a 10-year-old boy with hematuria, proteinuria, and mild to moderate bilateral sensorineural hearing loss (BSNHL), who was diagnosed with AS based on renal pathology and genetic testing. Whole exome sequencing of the proband identified a hemizygous canonical splice site variant (c.4994+1G>A) in intron 52 of the *COL4A5* gene. The same mutation was detected in his affected brother and heterozygous mother by Sanger sequencing, confirming the diagnosis of XLAS in the two affected individuals with renal impairment in this family. Our findings expand the geographic and mutational spectrum of *COL4A5* splice site variants. To the best of our knowledge, this is the first reported c.4994+1G>A variant (ClinVar: rs2524654509) in an Iranian family, underscoring the need for including splice site analysis in diagnostic testing.

Keywords: Alport Syndrome, *COL4A5* Protein, Human, RNA Splice Sites, Collagen Type IV, Exome Sequencing

1. Introduction

Alport syndrome (AS) is a genetically heterogeneous disorder characterized by progressive renal disease, sensorineural hearing loss, specific ocular abnormalities, and other less common extrarenal manifestations that result from disease-causing mutations in *COL4A3*, *COL4A4*, or *COL4A5* genes, encoding the α3, α4 and α5 chains of collagen type IV, respectively, which normally present in basement membrane of the kidney, cochlea, and eye (1).

The prevalence of AS is estimated to be from 1 in 5,000 to 1 in 53,000 of the population, thus making AS the second most common inherited kidney disorder after autosomal dominant Polycystic kidney disease (2, 3). AS can be inherited in four patterns: X-linked (XLAS), Autosomal recessive (ARAS), Autosomal dominant (ADAS), or Digenic. XLAS, caused by *COL4A5* mutations, is the most prevalent. ARAS and hemizygous males with XLAS exhibit more severe phenotypes, whereas ADAS and heterozygous females with XLAS display less severe symptoms and a broader spectrum of

symptoms. It is well established that a stronger genotype–phenotype correlation exists in males with XLAS (4).

Diagnosis of AS can be made by molecular genetic testing, skin biopsy, or kidney biopsy. Genetic testing is the gold standard in making a definitive diagnosis of AS due to its non-invasive nature and high sensitivity and specificity. In recent years, whole-exome sequencing via next-generation sequencing (NGS) has become the primary approach for genetic analysis of AS. Early definitive diagnosis of AS through genetic testing offers significant advantages, including the potential for earlier treatment initiation, prediction of renal disease progression and the onset of end-stage renal disease (ESRD), as well as genetic counseling (5).

In the current study, we report a canonical splicing variant (c.4994+1G>A) in the *COL4A5* gene causing XLAS. This variant is classified as pathogenic in ClinVar (rs2524654509) but has not been previously reported in Iranian patients (6). Published molecular data on Alport syndrome in Iranian populations are limited. Our report of this variant in two affected siblings from northwestern Iran provides population-specific insights into the molecular epidemiology of XLAS and supports the inclusion of intronic regions in diagnostic panels.

2. Case Presentation

2.1 Study Participants and Clinical Assessment

The proband (II-1) was a 10-year-old boy with a history of hematuria (onset at 3 years), proteinuria (onset at 7 years), and mild to moderate bilateral sensorineural hearing loss (BSNHL; onset at 8 years). He was the first child of non-consanguineous parents from northwestern Iran. Ophthalmologic examination was normal. He was referred to Watson Genetic Laboratory (Tehran, Iran) for genetic testing and counseling with a clinical and paraclinical diagnosis of AS. His mother (I-2, aged 35 years) had experienced hematuria and proteinuria during pregnancy, with no hearing loss or ocular abnormalities. His younger brother (II-2, aged 7 years) presented with hematuria at the age of 3 years, with audiometry and ophthalmologic assessments pending. A comprehensive medical history and physical examination were conducted, yielding relevant findings. Written informed consent was obtained, and the study was approved by the Ethics Committee at Zanjan University of Medical Sciences (IR.ZUMS.REC.1402.066) and the Watson Genetic Laboratory in Tehran, Iran, in accordance with the 1964 Helsinki Declaration.

A systematic assessment for extrarenal features was performed. The proband had confirmed bilateral sensorineural hearing loss; ocular examination results were normal. Data for the younger brother remains incomplete.

Finally, three participants (I-2, II-1, and II-2) were enrolled in this study (Figure 1). Clinical features, along with renal and hearing status and *COL4A5* genotypes, are summarized in Table 1.

2.2 DNA Extraction and Whole-Exome Sequencing

Genomic DNA was extracted from the patient's peripheral blood samples, as well as those of his mother and brother, using the Exgene™ blood SV mini-DNA purification kit (GeneAll Biotechnology, South Korea) according to the manufacturer's instructions. Human exome enrichment was performed on the proband (II-1) using the Twist Human Exome Plus kit (TWIST Bioscience, USA). The library was sequenced on the Illumina NovaSeq 6000 (Illumina, San Diego, CA, USA) by CeGaT GmbH, Germany, achieving a mean coverage depth of 100×. Sequence reads were aligned to the human reference genome (GRCh37/hg19), and variants were called and annotated using an in-house bioinformatics pipeline.

2.3 Variant Identification and Classification

WES identified a hemizygous canonical splice donor site variant; c.4994+1G>A (NM_033380, ChrX:107,938,670), in intron 52 of the *COL4A5* gene in the proband, predicted to cause reading-frame disruption. Several in silico tools, including CADD (Combined Annotation Dependent Depletion; score: 28.4, indicating high pathogenicity) and MutationTaster (prediction: disease-causing), supported the deleterious effect of the variant. Therefore, based on the American College of Medical Genetics and Genomics (ACMG) guidelines, it was classified as a pathogenic variant (criteria: PVS1, PM2, PP1) (7, 8).

Although in silico predictions strongly support pathogenicity, the absence of RNA-based functional assays (e.g., RT-PCR or minigene splicing analysis) represents a limitation, as these could confirm aberrant splicing and its downstream effects.

2.4 Variant Validation and Segregation Analysis

Variant confirmation and segregation analysis were performed by PCR-Sanger sequencing (ABI 3500; Pishgam Biotech Company, Tehran, Iran). Primer pairs were designed by Primer-Blast (NCBI), UCSC BLAT (http://genome.ucsc.edu/cgi-bin/hgBlat), and Gene Runner software (version 6.0) with the following sequences: Forward: 5'-GGTGTGGATACTATTGTCTTACCTCTG-3', Reverse: 5'-GCTATCACAAACCAAACTCACC-3'. PCR products were visualized on a 1.5% agarose gel electrophoresis before sequencing.

2.5 Limitations and Future Directions

Splice prediction was based on in silico tools; however, no RNA-based functional assays (e.g., RT-PCR or minigene analysis) were performed, which is a limitation. PCR-Sanger sequencing confirmed the *COL4A5*: c.4994+1G>A variant in a hemizygous state in the proband (II-1) and his symptomatic brother (II-2), and in a heterozygous state in their mother (I-2), establishing X-linked segregation.

This pathogenic variant (ClinVar: rs2524654509) segregated in two hemizygous brothers and their

heterozygous mother. Further cascade screening of maternal relatives is recommended to assess recurrence or potential founder status (Figure 2).

Table 1. Clinical features and *COL4A5* genotypes in the studied Iranian family with X-linked Alport Syndrome

Individual	Age	Genotype	Renal Manifestations	Hearing Assessment	Ocular Examination	Additional Features	Disease Severity
II-1 (Proband)	10y	Hemizygous c.4994+1G>A	Hematuria (onset 3y), Proteinuria (onset 7y), Normal GFR	Mild-moderate BSNHL (onset 8y), Bilateral, Progressive	Normal fundoscopy, No anterior lenticonus	Growth normal, No skin lesions	Moderate
II-2 (Brother)	3y	Hemizygous c.4994+1G>A	Hematuria (onset 3y), Proteinuria not yet detected	Audiometry pending	Ophthalmologic exam pending	Growth normal	Mild to Moderate
I-2 (Mother)	37y	Heterozygous carrier	Pregnancy-associated hematuria and proteinuria, otherwise asymptomatic	Normal hearing (audiometry performed)	Not examined	No extrarenal symptoms	Carrier, asymptomatic outside pregnancy

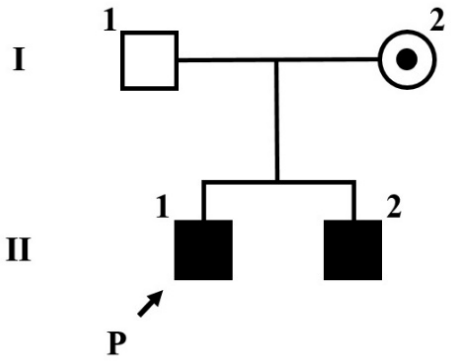

Figure 1. Pedigree of the family with X-linked Alport syndrome. An arrow indicates the proband. II-1 and II-2 are affected males; I-2 is a heterozygous carrier of the *COL4A5* mutation (**Designed by Authors, 2025**).

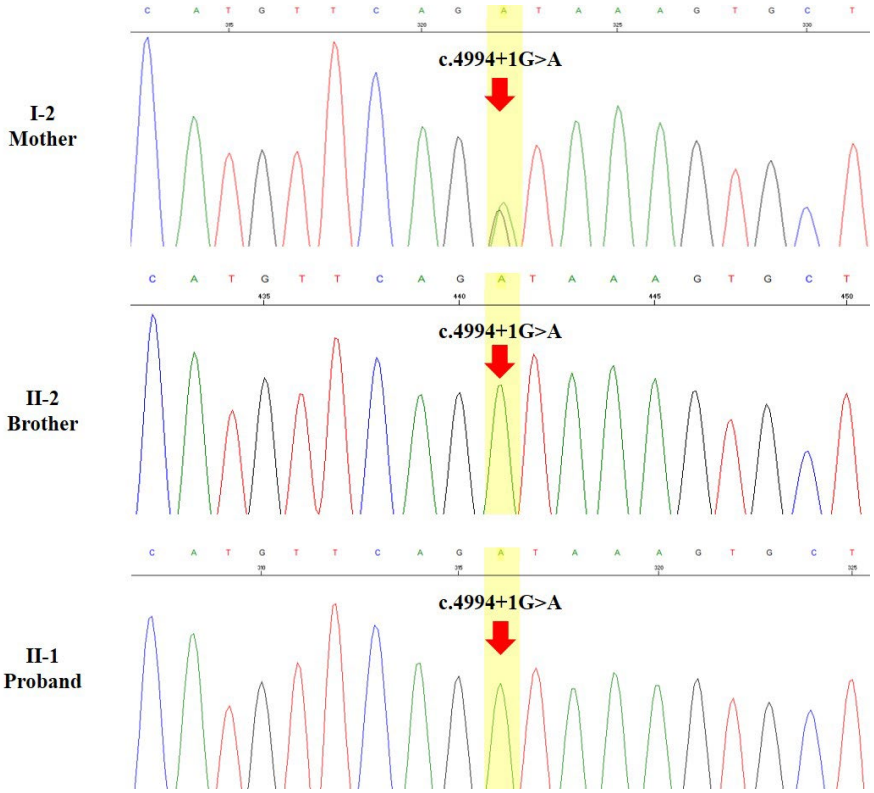

Figure 2. Survival and c patients (**Designed by Authors, 2025**).

3. Discussions

AS arises from disease-causing mutations in *COL4A3*, *COL4A4*, or *COL4A5* genes encoding collagen IV α chains. Family history and clinical screenings are strong evidence for the diagnosis of AS. However, the gold standard diagnosis is based on finding a pathogenic genomic mutation in *COL4A3*, *COL4A4*, or *COL4A5* genes. Previously, conventional PCR-Sanger sequencing was used for the genetic diagnosis of AS, although it was labor-intensive, time-consuming, and costly **(9)**. Additionally, certain conditions with a similar clinical presentation to AS, such as IgA Nephropathy, C3 glomerulopathy, and MYH9-related disorders, are not considered. Today, NGS-based methods, which have the potential to discover new mutations, provide a high-throughput and cost-effective approach for the early and accurate diagnosis of AS in regions like Iran, where molecular testing is limited **(5)**.

X-linked AS is the most frequent form of AS with pathogenic mutations in the *COL4A5* gene, which encodes the type IV collagen α5 chain. Affected males with this condition have hemizygous mutations in the *COL4A5* gene and severe manifestations. Approximately 60% of individuals start ESRD before the age of 30, and 90% by the age of 40. Approximately 90% of XLAS men have hearing loss before the age of 40, and about 30% of them have ocular abnormalities **(4)**.

Females with XLAS, who carry heterozygous mutations in *COL4A5*, show milder and variable phenotypes compared to males, which can be attributed to random X-inactivation and subsequent somatic mosaicism in females **(10)**. However, Yamamura et al **(11)** demonstrated that manifestations in XLAS females may be severe and associated with multifactorial mechanisms, although genotype couldn't predict the severity of the disease **(11)**.

To date, 486 missense, 77 nonsense, and 213 splicing mutations out of 1189 total mutations of the *COL4A5* gene have been reported in HGMD [https://www.hgmd.cf.ac.uk/ac/].

In the present study, we conducted whole-exome sequencing to find potentially disease-causing mutations (s) in an Iranian patient suspected of AS. Analysis of whole-exome sequencing revealed a hemizygous canonical splice site variant (NM_033380; c.4994+1G>A), which affects the donor site in intron 52 of the *COL4A5* gene associated with AS. Further family investigation revealed that his affected brother is also hemizygous for this mutation, and his mother is heterozygous. Both Proband and his brother had similar symptoms at the same age (3 years old), but their heterozygous mother had hematuria and proteinuria just during her pregnancies.

Although c.4994+1G>A is a recognized pathogenic variant (ClinVar: rs2524654509), previous reports involved non-Iranian individuals (ClinVar: SCV004406357.2 and SCV005683218.1) **(6)**. Our study is the first to document this splice site variant in an Iranian family, underscoring its regional relevance.

The detected mutation in the *COL4A5* gene is not anticipated to result in nonsense-mediated decay (NMD) but is predicted to truncate approximately 1.5% of the protein. The truncated fragment is critical to the protein structure and function. This region is located in the non-collagenous domain at the carboxyl terminus (NC1) of the collagen IV α5 chain. The NC1 domain serves as the initiation site for the assembly and cross-linking of the α3α4α5 heterotrimer through disulfide bonds between 12 highly conserved cysteine residues, ultimately leading to the formation of the collagen triple helix structure in glomeruli **(12)**. In addition, other pathogenic and likely pathogenic null mutations have been reported in the last intron and exon of the *COL4A5* gene (Figure 3).

About 11-14% of missense mutations in *COL4A5* affect the NC1 domain **(13)**. Although the clinical significance of these missense mutations is not yet well understood, many have been associated with later-onset renal failure in XLAS-affected males and various extrarenal manifestations **(12)**.

Comprehension of the genotype-phenotype correlation in males with AS increased the significance of discovering *COL4A5* splicing mutations, including canonical splice site variants, deep intronic variants, or single-base substitutions at the last nucleotide position in *COL4A5* exons **(14, 15)**.

Given the proportion of splicing variants in XLAS (~18%), inclusion of these junctions in routine panels and reflex RNA testing when WES is negative may improve detection rates **(16)**. The appearance of novel XLAS-therapeutic approaches affecting splicing mechanisms should be a focus in the future **(17)**.

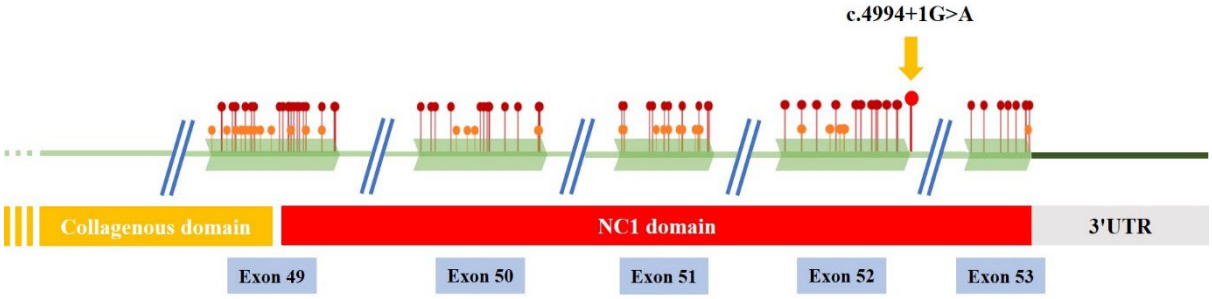

Figure 3. *COL4A5* pathogenic and likely pathogenic null variants located in the NC1 domain associated with XLAS previously reported in ClinVar (URL: https://www.ncbi.nlm.nih.gov/clinvar/). The red plot represents the detected variant in the proband.

Limitations

This study has several limitations. First, although the pathogenicity of the c.4994+1G>A variant is supported by ACMG criteria and multiple in silico tools, direct functional validation is lacking. RNA splicing assays, collagen IV expression studies in patient-derived or heterologous cell models, and electron microscopy of glomerular basement membranes would be valuable for confirming its impact on α3α4α5 heterotrimer assembly and testing the hypothesis that truncation of the NC1 domain disrupts cross-linking.

Second, the single-family design limits generalizability and may introduce bias. Unrecognized genetic modifiers or environmental factors unique to this Iranian kindred could influence phenotype severity, potentially overestimating penetrance or underestimating variability. Ascertainment bias is also possible, as recruitment was based on clinical suspicion of Alport syndrome. Broader studies in Iranian and regional cohorts would help clarify prevalence, genotype–phenotype correlations, and potential founder effects.

Finally, incomplete phenotypic characterization-such as pending audiometric and ophthalmologic evaluations for the younger sibling-limits the precision of clinical correlation in this report.

5. Conclusion

We report the first documented occurrence of the *COL4A5*: c.4994+1G>A splice-donor variant in an Iranian kindred with X-linked Alport syndrome. This finding expands the mutational spectrum of *COL4A5* in Iranian and regional populations, reinforcing the clinical value of interrogating splice junctions and intronic regions in molecular diagnostic panels. Its detection has direct implications for regional healthcare: enabling targeted cascade testing of at-risk relatives, integration of this variant into local and international databases, and provision of informed reproductive counselling, including preimplantation or prenatal genetic diagnosis where appropriate. In a setting where hereditary renal disorders may be under-recognized and access to comprehensive sequencing is limited, such case-level evidence supports the development of cost-effective, targeted testing strategies to facilitate early diagnosis and timely intervention. Furthermore, this report raises the possibility of a regional founder effect, warranting investigation in larger, population-based cohorts. Collaborative studies combining population screening with RNA-level functional assays will be essential to refine genotype–phenotype correlations and to inform precision diagnostic and therapeutic approaches for Alport syndrome in this population.

6. Declarations

6.1 Acknowledgments

The authors would like to extend their heartfelt gratitude to the patient and his family for participating in this study.

6.2 Ethical Considerations

All procedures performed in this study involving human participants were in accordance with the ethical standards of the Ethics Committee at Zanjan University of Medical Sciences, Zanjan, Iran (IR.ZUMS.REC.1402.066), and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

6.3 Authors' Contributions

A.A. was responsible for data analysis and manuscript preparation. M.K. supervised the project and reviewed the final version. All authors read and approved the final manuscript.

6.4 Conflict of Interest

The authors declare that they have no conflict of interest.

6.5 Fund or Financial Support

This study was funded by Zanjan University of Medical Sciences.

6.6 Using Artificial Intelligence Tools (AI Tools)

No AI-assisted tools were used in writing or analyzing the data for this study.

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How to Cite This Article:

Abyar A, Mohammadzadeh A, Dehghanzad R, Keramatipour M. A Canonical Splice Site Variant in the Last Intron of the *COL4A5* Gene Causing X-Linked Alport Syndrome in an Iranian Family: A Case Report. J Adv Med Biomed Res. 2025;33(159):341-7.

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