JBACHS
Journal of Basic and Clinical Health Sciences

J Basic Clin Health Sci 2019; 3:165-169
https://doi.org/10.30621/jbachs.2019.719

Original investigation

Initial Next-Generation Sequencing (NGS) Results of Alport Syndrome

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Received: 30.07.2019; **Accepted:** 06.08.2019; **Available Online Date:** 30.09.2019

Cite this article as: Koç Altuğ, Bora E, Cinleti T, Yıldız G, Torun Bayram M, Giray Bozkaya Ö, Ulgenalp A. Initial Next-Generation Sequencing (NGS) Results of Alport Syndrome. J Basic Clin Health Sci 2019; 3:165-169.

ABSTRACT

Objectives: We have no series report concerning genetic etiology of Alport Syndrome (AS) in our country. So, we aimed to investigate AS related pathogenic variants of *COL4A3*, *COL4A4* and *COL4A5* genes in index cases and their families who referred to our center.

Patients and Methods: The study includes 32 subjects (17 index cases and their relatives) who are investigated between years 2018-2019 by NGS targeting the coding regions of related genes. The test results and clinical findings of the cases are studied retrospectively.

Results: By the presented study, 19 individuals identified to have *COL4A3* and *COL4A5* variations which could be important for the clinical management. In four cases, there are novel variants. In two cases, there are digenic variations. There is no clinically relevant variant in *COL4A4* gene. The most frequent three mutations of *COL4A5* gene reported in United States (US) are not determined in our study group.

Conclusion: The diagnostic genetic tests of AS should be designed to include whole coding regions of *COL4A3* and *COL4A5* genes, not just for the frequently reported pathogenic variants. The cases without pathogenic variants by sequencing should be investigated for deletions/duplications of *COL4A5* gene. Clinical findings of our cases with novel genetic variants are presented as a contribution to literature.

Keywords: Alport syndrome, Hereditary Nephritis, COL4A3, COL4A4, COL4A5

INTRODUCTION

Pathogenic variants of *COL4A3*, *COL4A4*, *COL4A5* genes cause AS (also known as Hereditary Nephritis). It is a hereditary, progressive, glomerular disease. The syndrome may be presented as isolated hematuria with no progression or progressive renal disease (Table 1). Renal disease progresses in untreated cases, and it goes through the stages of microscopic hematuria, proteinuria, progressive renal insufficiency, and end stage renal disease (ESRD) (1, 2).

The prevalence of AS is 1/5,000. The disease is responsible for the 0.5% of the adults and 12.9% of the children with end stage renal disease (3, 4).

Pathogenesis of the AS is related with Type IV collagen which has 6 types of α chain (α1- α6). Triple helices formed by α chains constitute the basement membranes. *COL4A3*, *COL4A4*, *COL4A5* genes synthesize α3, α4 and α5 chains relatively and they are specifically constitute glomerular basement membranes (GBM), basement membranes of cochlea and ocular lens. If there is a pathogenic alteration in the genes coding α chain, the triple

Table 1. Diagnostic criteria for AS

I.	**Primary feature:**
	I-1. Persistent Hematuria
II.	**Secondary features:**
	II-1. Mutations in type IV collagen genes
	II-2. Type IV collagen abnormal expression
	II-3. Glomerular basement membrane (GBM) -specific abnormalities
III.	**Accessory features:**
	III-1. Family history of kidney diseases
	III-2. Bilateral sensorineural deafness
	III-3. Ocular abnormalities
	III-4. Diffuse leiomyomatosis

The main criterion is persistent hematuria. When patients fulfill one or more secondary features, or

two or more accessory features, in addition to the primary feature, they can be diagnosed with AS (3). AS: Alport syndrome.

helix structures are damaged and the result is nephropathy, sensorineural hearing loss and ocular lesions (3).

About two thirds of the AS cases have X linked (XL; *COL4A5*) inheritance, 20% of the cases have autosomal dominant inheritance (AD; *COL4A3* and *COL4A4*) and 15% have autosomal recessive (AR; *COL4A3* and *COL4A4*). Previously, it is regarded as AD inheritance pattern constitutes 5% of the Alport cases but recently, by the invention of NGS technologies, the ratio increases up to 20-30% for AD inheritance (2, 5). In AD Alport cases, ESRD is delayed to late adulthood, onset of hearing loss is relatively late and ocular disease is rare (1, 2). In addition to these patterns, there are digenic inheritance patterns for *COL4A3*, *COL4A4* and *COL4A5* genes (6, 7). Cases with somatic mosaicism are also reported (8). Family history of Alport cases frequently includes hematuria, deafness and renal insufficiency. Because, 10-15% of the XL genetic variations are *de novo*, some of the male patients and AR Alport cases may not have a family history with hematuria and renal disorder (1).

Bilateral, high frequency, sensorineural hearing loss and ocular anomalies accompany AS. Approximately, 80-90% of the male cases and some of the female cases with XL AS have hearing loss. Hearing loss is not congenital; typically, the onset is in late childhood or in early puberty which is diagnosed by audiometry. Ocular anomalies includes pathognomonic lenticonus, maculopathy, vesicles of corneal endothelium and recurrent corneal lesions (9). Large contiguous deletions of *COL4A5* and *COL4A6* genes may cause diffuse leiomyomatosis in some XL type Alport. Sole variations of *COL4A6* gene do not cause AS (10).

If the clinical findings of the AS are present, multigene panel testing including *COL4A3*, *COL4A4*, *COL4A5* genes and the genes included in differential diagnosis is appropriate. In cases with atypical phenotype, exome sequencing or microarray study may be useful (1).

To date, there is no definite treatment for AS. Therapeutic strategies aim to delay ESRD by using nephron protecting agents such as angiotensin converting enzyme inhibitors; and new treatment strategies are needed (11, 12).

We have case reports in literature for AS from our country but as far as we know there is no patient series (13). So we would like to present our initial genetic test results by this study.

MATERIAL and METHOD

Patient Selection

The subjects consist of the AS cases and their relatives that are referred for molecular genetic investigation to Department of Medical Genetics, Dokuz Eylul University between years 2018-2019. There are 32 individuals (17 males, 15 females) from 17 families (denoted as I to XVII). Mean age for the subjects is 19.2 years (range 1-60 years).

Sample Collection and DNA Extraction

Minimum 3 ml of peripheral blood samples of the subjects are collected to the EDTA tubes. The DNA extraction is done by the commercial QIAcube (Qiagen, Germany) according to offered extraction and purification protocols by the producer. The DNA is stored -20°C until NGS study.

Next-Generation Sequencing (NGS) of COL4A3, COL4A4 and COL4A5 Genes

DNA quality and concentration measurements are performed by Qubit (Thermofisher, USA) for the preparation of DNA samples to test kit (Celemics, South Korea). After the adjustment of proper DNA amount, library preparation step is done. In order, steps of the targeted NGS test kit includes: DNA fragmentation, purification, DNA end repair, purification, A-tailing, purification, adapter ligation, purification, indexing, purification, probe hybridization, targeted library selection by streptavidin beads, amplification of targeted library and purification according to the recommendations of producer. The library run is achieved by NGS platform (MiniSeq, Illumina, USA). The data delivered from NGS platform is analyzed by software Genomize Seq (V.16.7) (Genomize, Turkey). In addition, the ClinVar database (https://www.ncbi.nlm.nih.gov/clinvar/) and Varsome web tool (https://varsome.com/) are also used. The coding regions (± 10 base pair intronic) of *COL4A5* (ENST00000328300), *COL4A3* (ENST00000396578) and *COL4A4* (ENST00000396625) genes are investigated. For the pathogenicity interpretation and classification of genetic variants scoring system of ACMG recommendations are used (14).

RESULTS

The 13 individuals out of investigated 32 have no pathogenic variations. Three individuals have pathogenic (P), 3 have likely pathogenic (LP) and 13 have variant of unknown clinical significance (VUS) (Table 2). The distribution of detected variants among genes: *COL4A5* (3 P, 2 LP, 7 VUS) and *COL4A3* (1 LP, 4 VUS). Two cases (IX-1, IX-3) had VUS alterations in *COL4A5* and *COL4A3* genes at the same time. The allelic frequencies of 12 genetic variants which would have clinical consequences are presented in Table 3. The in-house and multi-center frequencies of P and LP variants determined by SEQ software are below 1%.

DISCUSSION

There are proposed diagnostic criteria for AS (Table 1). Seventeen index cases and their relatives, in total 32 individuals, are investigated by NGS analyses that are followed in pediatric genetics and nephrology because of AS. In literature, pathogenic variants are found in *COL4A5* (80-85%), *COL4A3* (12-15%) and *COL4A4* (5-8%) genes (1,3). Comparable results are obtained by our study: *COL4A5* pathogenic variants are the leading cause of AS and followed by *COL4A3* variations but there is no clinically meaningful *COL4A4* variation. In US, in some genetic centers frequent pathogenic variants of *COL4A5* are the sole targets of clinical genetic investigation: c.4692G>A (p.Cys1564Ser),

Table 2. The identified genetic variants and the clinical features of subjects are presented

Pathogenicity	Variant	Genotype	Family No.- Individual No.	Clinical Findings	Family History	*Relative, Gender, Age
Pathogenic	COL4A5 c.1871G>A (G624D), rs104886142	Heterozygote	IV-1	Recurrent fever, hematuria, proteinuria	Familial hematuria	Index, 12y, f
		Heterozygote	IV-2	-	Familial hematuria	Mother of IV-1, 40y, f
	COL4A5 c.2605G>A (G869R), rs104886189	Hemizygote	XI-1	Hematuria, minimal mesengial proliferation	-	11y, m
Likely Pathogenic	COL4A5 c.2689G>A (E897K), novel	Heterozygote	III-1	Hematuria, mild increase in mesangial matrix	-	4y, f
	COL4A5 c.3632G>A (G1211E), rs104886247	Heterozygote	VI-1	Hematuria, proteinuria, increased mesengial cellularity, uveitis	FSGS and renal transplantation in her father	7y, f
	COL4A3 c.442-2A>T (Splice Acceptor)	Heterozygote	XV-1	Hematuria, proteinuria, hearing loss	Hearing loss and hematuria in maternal uncle	13y, m
Variant of Unknown Clinical Significance (VUS)	COL4A5 c.1289C>A (A430D), rs142883891	Hemizygote	I-1	Antenatal hydronephrosis, nephrolithiasis, hematuria, proteinuria, anterior lenticonus	CRI and renal transplantation in maternal aunt	Index, 1.5y, m
		Heterozygote	I-2	-	-	Mother of I-1, 39y, f
		Heterozygote	I-4	CRI and renal transplantation	-	Maternal aunt of I-1, 45y, f
		Heterozygote	I-5	-	-	40y, f
		Heterozygote	I-6	-	-	34y, f
	COL4A3 c.1015G>C (G339R), novel	Homozygote	VIII-1	Hematuria, proteinuria, FSGS	CRI in ex. father	Index, 17y, m
		Heterozygote	VIII-2	-	-	Mother of VIII-1, 45y, f
	COL4A5 c.488T>C M163T, rs142503631 COL4A3 c.3338-3C>G (Intronic, Splice Region), novel	Heterozygote Heterozygote	IX-1	Hematuria	FSGS and hearing loss in father	Index, 10y, f
	COL4A3, c.3338-3C>G (Intronic, Splice Region), novel	Heterozygote	IX-2	Hematuria	FSGS and hearing loss in father	Brother of IX-1, 2.5y, m
	COL4A5 c.488T>C M163T, rs142503631 COL4A3 c.3338-3C>G (Intronic, Splice Region), novel	Hemizygote Heterozygote	IX-3	FSGS, hearing loss	-	Father of IX-1, 38y, m
	COL4A3 c.4270G>A (G1424R), novel	Heterozygote	XIII-1	Proteinuria	Proteinuria in cousin	10y, m
	COL4A5 c.5012C>T (T1671M), rs745360151	Heterozygote	XIV-1	Thick basement membrane in EM	Nephrolithiasis in father and mother	Index, 16y, f
	COL4A5 c.5012C>T (T1671M), rs745360151	Hemizygote	XIV-2	Intermittent hematuria, hearing loss (Trauma?)	-	Father of XIV-1, 60y, m

*If there are more than one family member the relationship is mentioned.
FSGS: focal segmental glomerulosclerosis; **CRI:** chronic renal insufficiency; **EM:** electron microscopy; **BM:** basement membrane; **Ex:** exitus; **y:** years; **m:** male; **f:** female.

Table 3. The in-house and national allele frequencies of variants are shown

Gene	Variant	Pathogenicity	In-house Allele Frequencies	SEQ National Allele Frequencies*
	c.5012C>T	VUS	0.016	0.001
	c.488T>C	VUS	0.011	0
	c.3632G>A	LP	0.004	0
COL4A5	c.2689G>A	LP	0.003	0
	c.2605G>A	P	0.005	0
	c.1871G>A	P	0.006	0.001
	c.1289C>A	VUS	0.02	0.007
	c.442-2A>T	LP	0.004	0.001
	c.4270G>A	VUS	0.003	0
COL4A3	c.3338-3C>G	VUS	0.014	0
	c.1015G>C	VUS	0.01	0
	rs886055736**	VUS	0.005	0

*The frequencies owing to centers using SEQ software (https://seq.genomize.com/).
**The variant is found in positive chain of COL4A3 gene and also negative chain of COL4A4 gene; so, the "rs number" is used.
VUS: variant of unknown clinical significance; **P:** pathogenic; **LP:** likely pathogenic.

c.4946T>G (p.Leu1649Arg), c.5030G>A (p.Arg1677Gln). The situation seems not true for our region, because none of them is seen in our study. At least, coding regions of the *COL4A5* and *COL4A3* genes are appropriate targets for us (1). In addition, deletions/duplications constitute 10-15% of pathogenic variants of *COL4A5* gene which can be detected by "Multiplex Ligation-dependent Probe Amplification (MLPA)" but not by sequencing (3). MLPA may be needed in cases with sequence negative test results.

It is reported that pathogenic variations of *COL4A4* gene are responsible for 5-8% of Alport cases (1), but we have no clinically important variation in *COL4A4* gene. This may be due to restricted number of studied individuals.

The results of the presented study are summarized in Table 2 but some families with special considerations are discussed separately.

Cases with Novel Variations

The clinical findings of three cases (III-1, VIII-1, and XIII-1) and their relatives with novel variations are discussed below:

Four years old girl (III-1) is referred with microscopic hematuria and non-nephrotic proteinuria. She has mild mesangial proliferation in her renal biopsy. Her hearing and ophthalmic examination are normal. *COL4A5* gene has heterozygous, pathogenic, novel variation c.2689G>A (E897K). Her family history does not include renal disease and her parents are not consanguineous. Her clinical findings are consistent with AS. Genetic family screening is planned.

The second case is 16 years old male patient (VIII-1). He has macroscopic hematuria and persistent microscopic hematuria, proteinuria and progressive renal insufficiency. His renal biopsy investigated by electron microscopy (EM) reveals irregular thick and thin areas in GBM. There is a novel, homozygous *COL4A3* VUS is determined in his genetic examination. The family history includes many individuals with chronic renal insufficiency (CRI) and consanguineous marriages. His father is dead because of CRI. The mother is a heterozygous carrier for the same *COL4A3* variation. So, the presented novel *COL4A3* variation c.1015G>C (G339R) is regarded as the cause of AS in this case.

The third case is 10 years old girl (IX-1). Her persistent microscopic hematuria and mild proteinuria are recognized when she was 1.5 years old for the first time. Her father (IX-3) has ESRD at 38 years and renal histopathology shows FSGS. Her brother (IX-2) has microscopic hematuria at 2.5 years old. Genetic investigation determines heterozygous VUS *COL4A3* c.3338-3C>G (intronic splice region) and *COL4A5* c.488T>C (M163T) in the patient and her father. The brother is heterozygous carrier for *COL4A3* variation. In the literature, the cases having both *COL4A5* and *COL4A3* variations have more sever clinic (7). So, close monitoring for the family is planned.

Case with Likely Pathogenic Variant

The case is 12 years old male (XV-1) and has microscopic glomerular hematuria, non-nephrotic proteinuria. The case has likely pathogenic *COL4A3* c.442-2A>T (Splice Acceptor) variation. The clinical findings of the mother and maternal uncle are consistent with AS and they have microscopic hematuria. The uncle also has hearing loss.

Cases with VUS

Clinical findings of three cases (I-1, XIII-1, and XIV-1) with VUS and their relatives are presented below:

The first case is 1.5 years old boy (I-1) with hemizygous VUS *COL4A5* c.1289C>A (A430D). The clinical findings do not

consistent with AS in his follow up. But his mother has the same variation and chronic renal disease also the renal transplantation in his family history leads us to follow family.

The second case is 9 years old boy (XIII-1). He has heterozygous *COL4A3* c.4270G>A (G1424R) variation. On his follow up transient microscopic hematuria, orthostatic proteinuria and by Doppler ultrasonography findings of Nutcracker syndrome are present. But, due to renal transplantation history of his cousin, close follow up is planned.

In third family, there is a daughter-father duo (XIV-1, XIV-2) with *COL4A5* VUS c.5012C>T (T1671M). The father is hemiygote and the daughter is heterozygote for the variation. Their clinical findings are as follows: The daughter (XIV-1) is 16 years old who has microscopic hematuria since 3.5 years. The EM findings from renal biopsy due to non-nephrotic proteinuria are consistent with AS. The father (XIV-2) has hearing loss and microscopic hematuria.

As a conclusion, the *COL4A3*, *COL4A4*, *COL4A5* genes which are responsible in the etiology of AS are investigated in presented report. The findings are comparable with the literature. Variants of *COL4A5* and *COL4A3* are detected in our series. These genes seem to be the main targets of genetic tests in our region but not the most frequent 3 pathogenic variations of US. MLPA should be considered as a complementary test in cases with negative sequencing result (15).

Our study reveals 4 families (III, VIII, IX, and XIII) with previously not reported variations out of 17 families. We define one likely pathogenic *COL4A5* variation c.2689G>A (E897K) and three VUS for *COL4A3* gene: c.1015G>C (G339R); c.3338-3C>G (Intronic, Splice Region); c.4270G>A (G1424R). Their clinical findings are identified.

Ethics Committee Approval: Dokuz Eylül University Non-invasive Research Ethics Committee 2019.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept - AK, AÜ; Design - EB, ÖGB; Supervision - AÜ; Materials - AK; Data Collection and/or Processing - TÇ, GY; Analysis and/or Interpretation - AK, E B, MTB, ÖGB, AÜ; Literature Search - AK; Writing Manuscript - AK; Critical Review - EB, M TB, ÖGB, AÜ

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declared that this study has received no financial support.

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