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Functional assessment of a novel *COL4A5* splice region variant and immunostaining of plucked hair follicles as an alternative method of diagnosis in X-linked Alport syndrome

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Abstract

Introduction—Many *COL4A5* splice region variants have been described in patients with X-linked Alport syndrome, but few have been confirmed by functional analysis to actually cause defective splicing. We sought to demonstrate that a novel *COL4A5* splice region variant in a family with Alport syndrome is pathogenic using functional studies. We also describe an alternative method of diagnosis.

Methods—We analyzed targeted next-generation sequencing results of an individual with Alport syndrome and confirmed results by Sanger sequencing in family members. A splicing reporter minigene assay was used to examine the variant's effect on splicing in transfected cells. Plucked hair follicles from patients and controls were examined for collagen IV proteins using immunofluorescence microscopy.

Results—A novel splice region mutation in *COL4A5*, c.1780-6T>G, was identified and segregated with disease in this family. This variant caused frequent skipping of exon 25, resulting in a frameshift and truncation of collagen $\alpha 5(\text{IV})$ protein. We also developed and validated a new approach to characterize the expression of collagen $\alpha 5(\text{IV})$ protein in the basement membranes of plucked hair follicles. We demonstrated reduced collagen $\alpha 5(\text{IV})$ protein in affected male and female individuals in this family, supporting frequent failure of normal splicing.

Conclusions—Differing normal to abnormal transcript ratios in affected individuals carrying splice region variants may contribute to variable disease severity observed in Alport families. Examination of plucked hair follicles in suspected X-linked Alport syndrome patients may offer a less invasive alternative method of diagnosis and serve as a pathogenicity test for *COL4A5* variants of uncertain significance.

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Disclosures

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Ethics statement

Human studies were conducted according to protocols approved by the Washington University Human Research Protection Office. Informed consent was obtained from all individual participants included in the study.

Keywords

Alport Syndrome; *COL4A5*; Splice site mutation; immunofluorescence; hair follicle

Introduction

Alport syndrome (AS) is a hereditary disease with a classical clinical presentation of renal failure, hearing loss, and ocular manifestations [1, 2]. The original descriptions of AS were in males who inherited disease in an X-linked Mendelian pattern, but AS can also be inherited in an autosomal fashion. The phenotype can vary in severity and age of presentation, both between and within families [3–6]. Early large-scale genetic studies of AS suggested a correlation between mutation type and phenotype [7, 8]. Hundreds of variants in the AS genes (*COL4A3*, *COL4A4* and *COL4A5*) have now been described, and approximately 13 – 16 % are splice site mutations [9]. However, the functional consequences of many splice region variants in AS patients have not been investigated. Furthermore, the correlation between genotype and phenotype at the molecular level has not been investigated for the vast majority of variants reported as pathogenic.

This report describes a novel splice region variant upstream of *COL4A5* exon 25 in a family with both males and females affected. We examined the consequences of this variant on exon 25 splicing using *in silico* and *in vitro* approaches. We also tested a novel non-invasive method to characterize the variable deposition of collagen $\alpha 5(\text{IV})$ protein in the basement membranes of plucked hair follicles from this family by simple immunofluorescence staining. This method could be generally applicable to help diagnose X-linked Alport syndrome.

Materials and methods

Patients and specimens

Patient recruitment and specimen collection and processing were performed by the Washington University Kidney Translational Research Core. Human studies were conducted according to protocols approved by the Washington University Human Research Protection Office. Informed consent was obtained from all individual participants included in the study.

Clinical data

A diagnosis of Alport Syndrome was made by kidney and/or skin biopsy in at least one individual in each generation of this kindred. All affected family members had documented persistent hematuria. Clinical histories and data were obtained from referring physicians after all documents were deidentified. Biopsies were clinically indicated and were performed by the treating physicians.

Custom targeted next-generation and direct Sanger sequencing

Targeted next-generation sequencing of a customized kidney disease gene panel was performed under IRB approval (Schulman IRB, PI: C. Larsen, Nephropath/Arkana Laboratories, Little Rock, AR) as a research test for one affected family member. This assay

used next-generation sequencing to analyze all coding exons and at least 10 bp of flanking intronic sequence of a panel of 301 genes to a minimum depth of X15 [10, 11]. Target enrichment was performed using oligonucleotide-based targeted capture (Agilent Rapid Capture Enrichment) of whole-genome shotgun sequencing libraries. Sequencing was performed on the Illumina MiSeq using a paired-end, 150 base-pair configuration. Analysis and interpretation was performed using DNASTAR software. All analyses were based on the human reference sequence UCSC build hg19 (NCBI build 37.2).

Sanger sequencing was performed on all family members from whom DNA was available. Primers flanking the splice region variant were used for PCR amplification and for forward and reverse sequencing. The primers were: COL4A5_SplMutG_Forward primer, 5'-TGGCTATATCCTTTCCCCAGT-3' and COL4A5_SplMutG_Reverse primer, 5'-GCCACACCTTGATGCCTTT-3'. Genomic DNA was amplified using the following temperature cycling protocol: 98°C for 3 min, [95°C for 1 min, 60°C for 45 sec, 72°C for 2 min] × 30 cycles, [95°C for 1 min, 55°C for 45 sec, 72°C for 2 min] × 30 cycles, 72°C for 10 mins then 4°C.

Splicing analysis

Firstly, we used publicly available *in silico* analysis tools to predict the effects of the c. 1780-6T>G variant on COL4A5 exon 25 splicing. These tools included Human Splicing Finder [<http://www.umd.be/HSF3/HSF.html>] [12], MaxEntScan::score3ss [http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq_acc.html] [13], and NetGene2 Server [<http://www.cbs.dtu.dk/services/NetGene2/>] [14,15].

Secondly, human COL4A5 exon 25 coding sequence plus 200–300 base-pairs of flanking intronic sequences were synthesized and inserted into the pCAS2.1 splicing vector by GenScript (Piscataway, NJ) using BamHI and MluI restriction sites, as described previously [16–18]. One vector contained the reference sequence (WT), and one contained the c. 1780-6T>G variant (VAR).

HEK293T cells were grown to 60 % confluence on 12-well plates and transfected in duplicate with either: no vector, empty pCAS2.1 vector, WT vector, VAR vector, or both WT and VAR vectors (2 ug total plasmid DNA per sample) using Lipofectamine 2000 reagent (Invitrogen). Cells were harvested 15–20 hours later, and RNA was extracted from each sample using microcolumn kitsTM with a DNase step (Qiagen). 1.5–2.0 ug of RNA were used to make cDNA using Multiscribe RT polymerase per protocol (AB systems). pCAS2.1 specific primers (pCAS-KO1-Forward primer: 5'-TGACGTGCGCCGCCCATCAC-3' and pCAS-2-Reverse primer: 5'-ATTGGTTGTTGAGTTGGTTGTC-3') were used to amplify cDNA to determine the splicing patterns. The amplicon should include COL4A5 exon 25 sequence if splicing function remained normal. To amplify cDNA we used KOD XtremeTM Hot Start DNA polymerase (per protocol), using three-step PCR cycling with a Tm of 53°C for 15 seconds per cycle for a total of 35 cycles.

Splicing patterns were determined by electrophoresis of PCR products on 1 % agarose gels. Bands of interest were purified for Sanger sequencing using the Wizard^R SV gel system (Promega).

Collagen IV $\alpha 2$ and $\alpha 5$ staining of hair follicles

Fluorochrome-conjugated monoclonal anti-human collagen IV antibodies (Cosmo Bio, Carlsbad, CA) were used to stain plucked hairs. A FITC-conjugated antibody to collagen $\alpha 5(\text{IV})$ imperfection III, the epitope of which is a five amino acid sequence (IDVEF) encoded by exon 13 [19], a FITC-conjugated antibody to collagen $\alpha 5(\text{IV})$ NC1 domain [20], and a Texas Red-conjugated antibody to collagen $\alpha 2(\text{IV})$ imperfection XIII [19] were incubated with hairs at room temperature for 30 minutes. Hairs were then whole-mounted for confocal immunofluorescence microscopy imaging of follicles.

Results

Alport family clinical findings

Family 68 is a three-generation pedigree with affected family members in each generation (Fig. 1 and Table 1). There was no male-to-male transmission of disease. No affected members of this family are known to have hearing loss or ocular abnormalities typical of Alport syndrome.

Mother (3226) and uncle (3) of the proband (3222) developed persistent hematuria in the first decade of life. The uncle (3) progressed to end-stage renal disease (ESRD) by the age of 14 years. At the time of submission the mother (3226) had chronic kidney disease (CKD) stage 3 and the maternal grandmother (3223) had preserved renal function. The proband (3222) and his brother (3227) developed hematuria and proteinuria by 5 years of age and have been on ACEi/ARB therapy since ages 8 and 7, respectively. At the time of manuscript submission both showed preserved renal function (serum creatinines of 1.0 and 0.8 mg/dL, estimated GFRs > 60 ml/min) at the ages of 21 and 19 years, respectively.

The proband was diagnosed with Alport syndrome after a kidney biopsy showing thin glomerular basement membranes (GBM) of 90–100 nm thickness with areas of lamina densa lamellation and a skin biopsy showing absence of collagen $\alpha 5(\text{IV})$ staining. All of the affected family members (except 3224 and 3225) had a skin biopsy to confirm their Alport syndrome diagnosis, with absence of $\alpha 5(\text{IV})$ staining in the male biopsies and segmental loss of $\alpha 5(\text{IV})$ staining in the female biopsies. Two female first cousins (3224 and 3225) of the proband were also diagnosed with Alport syndrome during childhood after the finding of hematuria in the setting of a family history of Alport Syndrome.

Germline genetic analysis

Patient 3227 had custom targeted next-generation sequencing performed. This revealed a novel splice region variant, c.1780-6T>G, in *COL4A5* intron 24. No other potentially pathogenic variants in any of the collagen IV genes associated with Alport syndrome (*COL4A3*, *COL4A4* and *COL4A5*) were found. Sanger sequencing confirmed that the c.1780-6T>G variant segregated with disease (Fig. 1). This variant was not found in the unaffected sibling (3232) of the proband and was not found in the married-in father (3231) of the proband. One heterozygous variant in complement gene *CFI*, c.782G>A, was found. This has a minor allele frequency of <1 % but has been previously described (rs112534524),

and studies by Nilsson et al. found that this variant is not likely to be pathogenic [11]. No other variants in the genes included in the panel were found.

In silico splicing analysis

The c.1780-6T>G variant in *COL4A5* was predicted to have widely variable impact on splicing, depending upon the specific *in silico* algorithm used. At one extreme, Human Splicing Finder detected no significant impact on splicing. MaxEntScan::score3ss predicted 13.2 % reduced splicing efficiency. NetGene2 Server predicted a 75 % drop in confidence that splicing would occur when the variant was present. The inconsistencies in these predictions precluded defining the variant as pathogenic.

In vitro splicing analysis

Functional assessment of the WT and c.1780-6T>G splice region variant was performed in cultured cells using the pCAS2.1 splicing reporter minigene assay (see Materials and Methods). The WT and VAR plasmids were transfected into HEK293T cells both separately and together. RNA was prepared from the cells and subjected to RT-PCR using amplification primers targeting the vectors' exonic sequences flanking the *COL4A5* insert (Fig. 2A). Agarose gel electrophoresis (Fig. 2B) showed that cells transfected with the WT vector yielded the expected 404 bp amplicon containing the vector's endogenous exons and the *COL4A5* exon 25 between them, with the intronic sequences spliced out. In contrast, cells transfected with the VAR vector yielded a major amplicon of 235 bp lacking *COL4A5* exon 25 and a minor amplicon of 404 bp. Cells transfected with both WT and VAR vectors also yielded amplicons of 404 bp and 235 bp, but the 404 bp band was brighter than the 235 bp band (Fig. 2B, wt + var). The 404 bp band contained *COL4A5* exon 25 (169 bp) flanked by the exonic sequences intrinsic to the pCAS2.1 splicing vector (exons 2 and 3 of *C1NH*); the 235 bp band represents the spliced pCAS2.1 exons without *COL4A5* exon 25 (Supplemental Figure 1). Thus, the c.1780-6T>G variant caused frequent skipping of exon 25 in HEK293T cells, indicating that it is pathogenic.

Analysis of collagen IV expression in hair follicles

Because the epidermal basement membrane is continuous with the hair follicle basement membrane, we reasoned that analysis of collagen IV in plucked hair follicles could be a new method to help diagnose Alport syndrome noninvasively. Hair follicles plucked from affected and married-in members of Family 68 and from controls were stained for collagen $\alpha 2(\text{IV})$ and $\alpha 5(\text{IV})$ proteins by direct immunofluorescence. There was reduced and abnormal $\alpha 5(\text{IV})$ staining in the follicles of affected individuals (Fig. 3B, C) compared to control follicles (Fig. 3A) and follicles from a married-in family member (Fig. 3D). We noted that hairs plucked from affected individuals were less likely to have basement membrane attached to the hair root compared to unaffected individuals and controls.

Discussion

This study describes a novel *COL4A5* variant in an Alport Syndrome family in which both males and females are affected. Males tended to have more severe disease than females, suggesting the classic X-linked pattern of inheritance. This disease severity difference

between the sexes is demonstrated by individual 3 who progressed to ESRD by the age of 14 versus his affected sister (individual 3226) and mother (3223) who had not progressed to severe renal failure by the ages of 55 and 75, respectively.

All of the affected members of this family who were tested carried the c.1780-6T>G splice region variant in *COL4A5*, but the unaffected sibling and married-in father of the proband did not. Thus, this variant segregates with disease and is highly penetrant. Initial examination of this variant using online *in silico* tools suggested the variant might be pathogenic, but results from different algorithms were inconsistent. This is frequently the case for variants that are subsequently shown to be pathogenic using *in vitro* or *in vivo* models. For this reason we tested the functional consequences of this variant using a simple *in vitro* splicing reporter minigene assay. The c.1780-6T>G variant caused frequent yet apparently incomplete exon skipping (Fig. 2 and Supplementary Figure 1). RT-PCR data from the variant vector showed both skipped exon 25 product and retained exon 25 product; however, the predominant product lacked exon 25. When equal amounts of wild type and variant vector were co-expressed, the retained exon 25 message was the predominant transcript detected.

A particularly interesting finding in this study is that the variant vector can express some normally spliced transcript, at least in transfected HEK293T cells. Although other groups have reported using RNA extracted from hair follicles to assay *COL4A5* expression and/or mRNA splicing by RT-PCR [21–23], we were unable to obtain enough intact RNA to determine the extent to which normal splicing of exon 25 occurs *in vivo*. However, if a low frequency of exon 25 retention does occur *in vivo*, it could partly explain the differences in disease severity among the affected males in this family (e.g., individual 3 compared to individuals 3222 and 3227), and it may also partly explain differences in phenotype between affected females (e.g., individual 3223 who has preserved renal function and her daughter, individual 3226, who has CKD stage 3).

In this regard, a recent article on females and Alport syndrome highlights the increased risk of disease, variable phenotype-genotype correlations in females with X-linked Alport mutations, and the importance of Lyonization, the random inactivation of one X chromosome [24]. This adds further complexity to the diagnosis and management of Alport syndrome in females with *COL4A5* mutations. The extent of inactivation of the normal X chromosome in the affected females, in addition to the extent of *COL4A5* exon 25 retention, may further increase the degree of phenotypic variation between affected females. Analysis of X inactivation in this family was not performed. The presence of unidentified modifier gene variants in this family may also explain variation in phenotype within this and within other families with Alport syndrome.

As *COL4A5* exon 25 is 169 base pairs in length, its loss causes a frameshift and truncation of the $\alpha 5(\text{IV})$ protein. Gross et al. have previously reported that males with X-linked AS caused by a frame shift mutation progress to ESRD at a mean age of 19.8 ± 5.7 years [25]. On the other hand, mild renal disease in a male, despite carrying a splice region variant that causes a truncation, has also been described before. Nozu et al. demonstrated the presence of wild type and abnormal transcripts in the kidney of an affected male with a splice region

variant in *COL4A5* that causes a truncation [21]. Our findings also suggest that incomplete splicing abnormalities might occur in the kidney and lead to a variable phenotype.

The incomplete skipping of exon 25 shown in this study would be expected to lead to reduced and variable protein deposition in tissues where *COL4A5* is expressed. As *COL4A5* is expressed in the skin, we examined the basement membranes of plucked hair follicles. Both $\alpha 2$ (in $[\alpha 1(\text{IV})]_2\alpha 2(\text{IV})$) and $\alpha 5$ (in $[\alpha 5(\text{IV})]_2\alpha 6(\text{IV})$) proteins are normally present in hair follicles [26]. Using the above described novel and non-invasive method we found that $\alpha 5(\text{IV})$ protein expression was reduced in the basement membranes of hair follicles of affected family members compared to controls. We also found that hair plucked from affected family members frequently lacked basement membrane compared to hairs plucked from controls (data not shown). This finding may be explained by the discontinuity of the lamina densa of skin basement membranes in AS patients [27].

Demonstration of a reduction or loss of $\alpha 5(\text{IV})$ expression in hair follicle basement membranes is a potentially useful and non-invasive method of diagnosis in AS. In this regard it is important to note that alternative splicing of *COL4A5* RNA has been shown to generate two mRNAs (t1 and t2) and that hair follicles contain only t1, which lacks two small exons of 9 bp each. To our knowledge, no mutations have been described in these exons, but a mutation impacting these exons could cause kidney disease without altering expression of *COL4A5* in hair follicles. With this caveat in mind, this $\alpha 5(\text{IV})$ protein assay is novel and has the potential to be a clinically meaningful screening tool for Alport syndrome. It would be especially valuable in determining the functional consequence of variants that would otherwise be described as ‘variants of uncertain significance’. Further studies on a large cohort of Alport families representing the full spectrum of mutation types is needed to determine the clinical utility of this hair follicle assay.

Reduction and variable expression of $\alpha 5(\text{IV})$ protein in glomeruli could not be examined here due to lack of specimens. However, our findings in the basement membrane of hair follicles suggest that there is likely reduced and/or variable $\alpha 3\alpha 4\alpha 5(\text{IV})$ protein expression in the GBM. Reduced but not absent $\alpha 5(\text{IV})$ protein in the GBM of males with X-linked AS has been described before. Only one of the $\alpha 5(\text{IV})$ -positive patients described by Hashimura et al. had a splice site mutation; however, we know from Nozu et al. that splice region variants can lead to expression of both normal and abnormal transcripts in the kidneys of male patients with X-linked AS [21, 28].

The amount of normal *COL4A5* transcript expressed *in vivo* by the c.1780-6T>G variant may vary significantly between cell types. For example, keratinocytes may express no normal transcript, and thus would explain the skin biopsy results in affected family members showing loss of $\alpha 5(\text{IV})$ protein. It is interesting to speculate that the relevant cells in the inner ear that normally express *COL4A5* are able to properly splice exon 25 to some extent, resulting in the preserved hearing function observed in this family.

In summary, this study reports a novel splice region variant in intron 24 of *COL4A5*, c.1780-6T>G, in a family with X-linked Alport syndrome. We used an *in vitro* assay to show that this variant is a pathogenic mutation that causes frequent but incomplete skipping of

exon 25. This mutation likely leads to expression of mostly abnormal but also some normal transcripts that may partially explain the variable intra-family phenotypes seen in individuals carrying the mutation. Furthermore, we describe a novel non-invasive method to characterize $\alpha 5(\text{IV})$ protein deposition in the basement membranes of plucked hair follicles that may be an alternative method of diagnosis for Alport syndrome.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Pedigree of Alport Family 68 with chromatograms showing that the c.1780-6T>G splice region variant is present in affected but not unaffected family members

Squares, males; circles, females; open shapes, unaffected; filled shapes, affected; diagonal strike, deceased. The numbers beneath the icons are individual identification numbers and correspond to the respective chromatograms. The heterozygous variant in females shown as overlapping peaks in the chromatograms is denoted by brackets.

Figure 2. *In vitro* analysis of the splice region variant's function in HEK293T cells reveals frequent skipping of exon 25

(A) The diagram illustrates the relevant intronic (dashed lines) and exonic (boxes) regions of the splicing reporter minigene vector. Black boxes represent C1NH exons 2 and 3, and the open box represents exon 25 of *COL4A5*. The c.1780-6T>G splice region variant is indicated. (B) RT-PCR products obtained from cells transfected with wild type (wt) vector only, variant (var) vector only, or wt and var vectors together. Product sizes are indicated based on size markers in lane 4. Diagrams to the left represent the normally spliced transcript containing exon 25 (top) and the abnormally spliced transcript resulting from the c.1780-6T>G variant (bottom).

Figure 3. Immunofluorescence staining of plucked hair follicle basement membranes from a control and from Alport family members

FITC-conjugated antibodies to collagen $\alpha 5(\text{IV})$ (green) and Texas Red-conjugated antibody to collagen $\alpha 2(\text{IV})$ (red) were used. A, Control follicle, *COL4A5*^{WT/WT}. B, 3223 follicle, *COL4A5*^{c.1780-6T>G/WT}. C, 3227 follicle, *COL4A5*^{c.1780-6T>G/Y}. D, 3231 follicle, *COL4A5*^{WT/Y}. COL4A2 staining shows where basement membrane is present. COL4A5 was abundant in the control (A) and unaffected family member (D) follicle basement membranes but absent (B) or weak/segmental (C) in those with the variant.

Table 1

Clinical features of Alport family 68

Subject ID	Pedigree	Sex	Diagnosis
3223	I.1	Female	Alport. Hematuria, proteinuria Skin Biopsy: Segmental loss of $\alpha 5$ staining
3226	II.1	Female	Alport. Hematuria, proteinuria before age 10 Progressed to CKD 3 by age 55 Skin Biopsy: Segmental loss of $\alpha 5$ staining
3231	II.2	Male	Unaffected
3	II.3	Male	Alport. Hematuria, proteinuria before age 10 ESRD age 14. Transplant at age 15+17
3224	III.1	Female	Alport. Hematuria
3225	III.2	Female	Alport. Hematuria
3227	III.3	Male	Alport. hematuria, proteinuria age 5 Skin Biopsy age 7: Absence of $\alpha 5$ staining Wilsons disease
3222	III.4	Male	Alport. Hematuria, proteinuria age 5 Kidney biopsy age 7: Thin GBM 90–100nm Skin Biopsy: Absence of $\alpha 5$ staining
3232	III.4	Female	Unaffected Skin biopsy: Normal $\alpha 5$ staining

CKD–Chronic kidney disease; ESRD–End-stage renal disease; GBM–Glomerular basement membrane