Alport Syndrome

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Jane W. Kimani and Karen E. Weck

Clinical Background

A.K. was a 5-year-old boy who presented to the pediatric nephrology clinic with a recent finding of microscopic hematuria and proteinuria on routine screening. The analysis was repeated two weeks later with persistence of hematuria and proteinuria. A complete blood count (CBC) and a metabolic panel (Chem-7) were both normal. Renal ultrasound was performed which was also normal and without hydronephrosis. A.K. had one younger brother who was two years old with no health problems. A.K.'s father was 38 years old and had no health concerns. A.K.'s father's brother, sister, and parents were all healthy, with no renal concerns. A.K.'s father's brother had one son who was healthy at seven years. A.K.'s mother was healthy at 37 years. She had one brother and two sisters, none of whom had any renal concerns. One of her sisters had a son and a daughter; the son, who was six years old, had proteinuria found on dipstick about a year ago, but he has not been referred to a nephrologist. A.K.'s maternal grandfather was healthy and his grandmother died of myocardial infarction at the age of 60.

Question 1: Draw a three-generation pedigree for this family
Question 2: What is your differential diagnosis?

Reason for Molecular Testing

A diagnosis of X-linked Alport syndrome (XLAS) was suspected. Diagnosis of Alport syndrome is complex and requires urinalysis, renal function studies, audiometry, ophthalmic evaluation, and skin and/or kidney biopsy. Molecular testing for mutations in the *COL4A5* gene is useful for diagnosis of XLAS as other diagnostic methods may be inconclusive in the early stages of renal disease. Molecular testing is also useful for prognosis, as identification of specific mutations may be helpful to predict disease severity. In addition, molecular testing is useful for family testing to identify other male relatives who are at risk of developing symptoms and to identify female carriers. Finally, while renal transplantation is an effective treatment for Alport syndrome, identification of an unaffected living-related donor can be difficult and can be guided with molecular testing in families who have a known mutation.

Test Ordered

The physician ordered molecular testing for *COL4A5*.

J.W. Kimani (✉)
Department of Pathology and Laboratory Medicine,
University of North Carolina at Chapel Hill,
CB#7600, 101 Manning Drive, Chapel Hill,
NC 27514, USA
e-mail: jkimani@unch.unc.edu

K.E. Weck
Departments of Pathology & Laboratory Medicine and
Genetics, University of North Carolina at Chapel Hill,
Brinkhous-Bullitt Building, Campus Box 7525,
Chapel Hill, NC 27599-7525, USA
e-mail: kweck@unc.edu

I. Schrijver (ed.), *Diagnostic Molecular Pathology in Practice*,
DOI: 10.1007/978-3-642-19677-5_2, © Springer-Verlag Berlin Heidelberg 2011

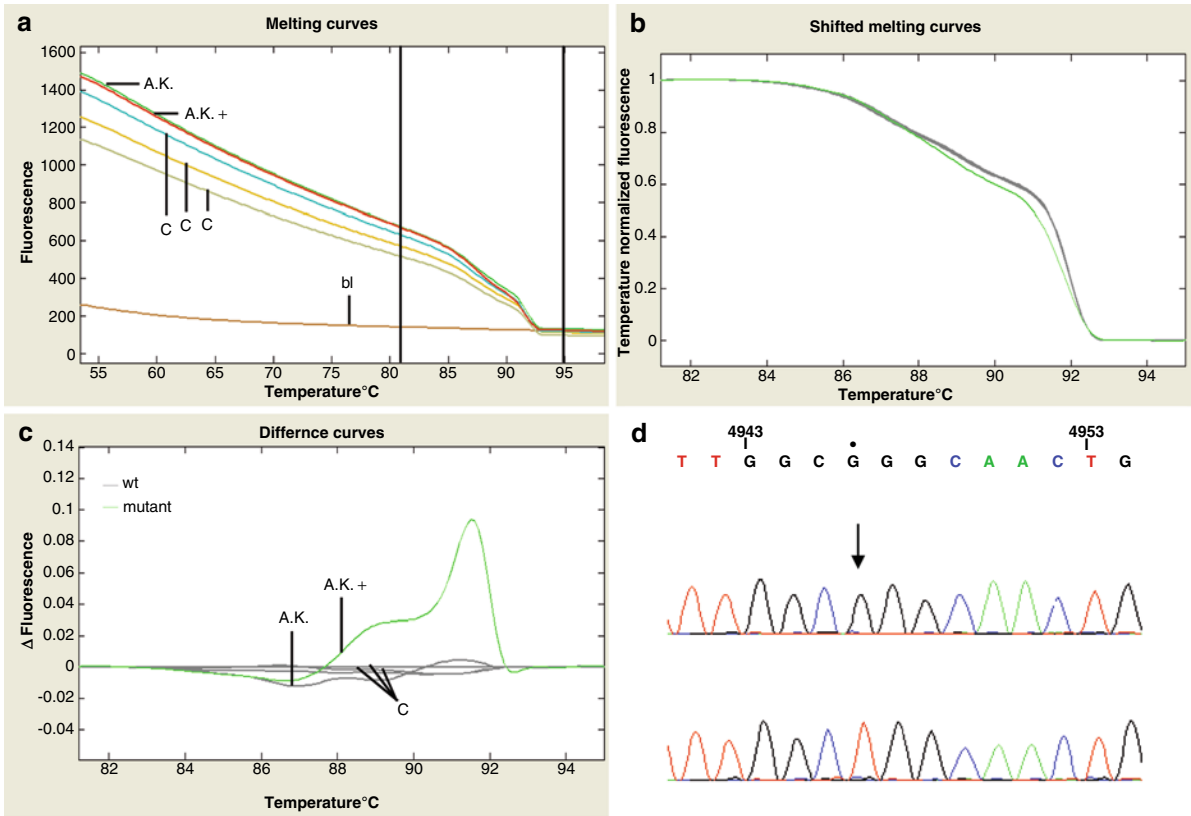

Fig. 2.1 High resolution melting curves and partial DNA sequencing analysis for *COL4A5* exon 50. (**a**) Fluorescence (*F*) *versus* temperature (*T*) melting curves using raw fluorescence data. (**b**) Temperature shifted melting curves after fluorescence normalization. (**c**) Fluorescence difference curves. (**d**) Sequencing electropherograms showing patient sample A.K. (*top panel*) and a control wild-type sample (*bottom panel*). *A.K.* patient sample (neat), *A.K.+* patient sample spiked with normal DNA, *C* control wild-type samples, *bl* blank (no template control)

Laboratory Test Performed

Mutation scanning of the exons and flanking intronic regions of the *COL4A5* gene was performed using high resolution melting analysis (HRMA) followed by DNA sequencing of any exons with an abnormal melting profile. *COL4A5* is a large 51-exon gene that spans a genomic region of approximately 250 kb on chromosome Xq22 and generates an RNA transcript of about 6.5 kb. There is no mutation hotspot and hundreds of mutations, most of them missense mutations, have been identified throughout the gene. Molecular diagnosis therefore requires analysis of the entire coding region either by direct sequence analysis or mutation scanning followed by sequence analysis of exons with putative sequence variation.

Question 3: What are the limitations and advantages of this approach?

Results with Interpretation Guideline

The results of mutation scanning by HRMA of the *COL4A5* gene demonstrated an abnormal melting profile for exon 50 (Fig. 2.1); the HRMA results for all other exons were normal.

HRMA detects sequence variation in a DNA fragment based on differences in melting properties relative to a normal control (wild-type) sample. In our case, individual exons were amplified by PCR in the presence of a saturating DNA-binding dye such as LCGreenPlus that fluoresces only in the presence of

double-stranded DNA. The PCR was followed by a "heteroduplex formation" cycle involving denaturation at 94°C for 30 s, followed by cooling to 25°C for 30 s. The amplicons were then melted slowly on a LightScanner instrument (Idaho Technology Inc., Salt Lake City, UT) by increasing the temperature to 96°C at a rate of 0.1°C/s. The decrease in fluorescence was measured as the double-stranded DNA molecules melt apart.

Figure 2.1a shows the decrease in fluorescence as a function of increasing temperature as the double-stranded DNA molecules labeled with LCGreenPlus dye melt apart for three normal control (C) samples, the patient sample (A.K.), the patient sample mixed in a 1:1 ratio with a wild-type control sample (A.K.+), and a no template water control (bl). Figure 2.1b reflects the melting curves from 81°C to 95°C after fluorescence normalization by Call-IT™ software (Idaho Technology Inc., Salt Lake City, UT). Figure 2.1c demonstrates the difference in the melting curve of each sample compared to a normal control sample. The Call-IT™ software groups samples based on the similarity of the melting curve to the normal control (shown in *gray*). Samples with significant difference in melting profile from the normal control are grouped as unknowns (shown in *green*). The neat patient sample (A.K.) clusters with the wild-type control samples, but the spiked patient sample (A.K.+) demonstrates an abnormal melting curve. This result illustrates the increase in sensitivity of HRMA for detection of a hemizygous (e.g., X-linked) mutation by mixing with normal DNA. This forces heteroduplexes of normal and mutant DNA molecules which melt more easily than homoduplexes of identical DNA molecules. DNA sequencing of *COL4A5* exon 50 was subsequently performed to identify the mutation (Fig. 2.1d).

Result Interpretation

Mutation scanning by HRMA followed by DNA sequencing revealed that the patient has a c.4946T>G (p.Leu1649Arg) mutation in the *COL4A5* gene. A single nucleotide at position 4946 of the cDNA was changed from a thymine (T) to a guanine (G). In the primary protein structure, this missense mutation results in the substitution of a leucine codon (CTG) at position 1649 by an arginine codon (CGG). This *COL4A5* L1649R mutation substitutes a conserved neutral amino acid in the non-collagenous (NC1) domain of the COL4A5 protein with a charged amino acid. This mutation has previously been reported in patients with Alport syndrome [1]. The results are consistent with a diagnosis of Alport syndrome.

Question 4: Does this result explain the patient's symptoms?

Table 2.1 Types of Alport syndrome based on the genes involved and the inheritance pattern

Mode of inheritance	Genes	Frequency (%)
X-linked	*COL4A5*	80
Autosomal recessive	*COL4A3* and *COL4A4*	15
Autosomal dominant	*COL4A3* and *COL4A4*	5

Further Testing

There is no need for further genetic testing of the patient. However, his kidney function should be monitored closely for disease progression to allow timely treatment and intervention. It is also recommended that he be referred to an ophthalmologist and audiologist for assessment of extra-renal manifestations of Alport syndrome. The identification of a disease-causing mutation in A.K. allows for molecular diagnostic testing of at-risk family members. Targeted testing of *COL4A5* exon 50 in A.K.'s mother revealed the c.4946T>G (p.L1649R) mutation in a heterozygous state, confirming that she is a carrier of XLAS. Genetic testing is recommended for the maternal cousin with proteinuria and for A.K.'s younger brother if he develops symptoms of Alport syndrome such as hematuria.

Background and Molecular Pathology

Alport syndrome (OMIM # 301050) is a heterogeneous disorder characterized by progressive renal disease, cochlear, and ocular defects. It has an estimated prevalence of approximately 1:50,000 live births [2]. Mutations in the type IV collagen genes that code for structural components of basement membranes are the underlying cause of Alport syndrome. There are three types of Alport syndrome as shown in Table 2.1.

Alport syndrome is predominantly an X-linked disease. Males present with persistent microscopic

and episodic gross hematuria from childhood, which develops into proteinuria, progressive renal insufficiency, and eventually end stage renal disease (ESRD). Other symptoms including progressive hearing loss and ocular lesions, particularly anterior lenticonus, may be present depending on the underlying mutation. However, there can be variability in the age of onset even in family members with the same mutation [3]. Clinical features in females vary from severe involvement, intermittent microscopic hematuria, to no symptoms at all. Hearing loss and ocular lesions are infrequent in female carriers. The clinical features of autosomal recessive Alport syndrome are similar to those of X-linked Alport syndrome in males, but affect males and females equally. Autosomal dominant Alport syndrome has a variable clinical phenotype that is generally milder than both X-linked and autosomal recessive Alport syndrome [4].

There are six genetically distinct type IV collagen alpha chains (α1–α6) that together with other molecules such as laminins and proteoglycans form structural components of basement membranes. The basement membrane is a sheet-like structure found between the epithelium and the tissue stroma that provides cellular support, compartmentalizes tissues, and is involved in various biological functions including growth and differentiation, tissue repair and molecular ultra-filtration. Each type IV α-chain consists of a middle triple-helical domain with the characteristic collagenous Gly-X-Y motif, flanked by an amino-terminal 7S domain and a carboxy-terminal non-collagenous (NC1) domain. The α1(IV) and α2(IV) chains have ubiquitous expression in all basement membranes, but the expression of α3(IV), α4(IV), and α5(IV) chains is specific to the basement membranes of the glomerulus, the inner ear, and the corneal epithelium. Three α-chains initiate assembly at the NC1 domain to form triple helical protomers, which form the building blocks for the self-assembly of a collagen type IV supra-structure network [5, 6].

COL4A5 mutations result in defective or deficient α5(IV) chains, which also abolishes expression of the α3(IV) and α4(IV) chains. This causes ultrastructural changes in the glomerular basement membrane (GBM) such as irregular thinning and thickening that can be observed by electron microscopy in renal biopsy specimens from affected patients. There is no mutation hotspot within the *COL4A5* gene and recurrent mutations are rarely seen. Hundreds of mutations have been reported throughout the gene including missense (40–48%), splice site (11–16%), nonsense and frameshift (25–30%), and large rearrangement (6–20%) mutations. The incidence of *de novo* mutations is 3–12% [4, 7]. The missense mutations mostly involve substitution of the glycine residue within the Gly-X-Y motif with a bulkier amino acid, which alters the secondary structure of the protein resulting in defective assembly of the corresponding α-chain. Genotype–phenotype correlations in Alport syndrome are not well established. However, large gene rearrangements, nonsense, and frameshift mutations that result in a truncated or absent protein are generally associated with a more severe phenotype and earlier onset of ESRD, compared with missense mutations. Additionally, because assembly of the collagen protomers begins at the carboxy-terminal NC1-domain, glycine missense mutations involving the 3′ end of the gene generally result in a more severe phenotype than those involving the 5′ end of the gene [8].

Multiple Choice Questions

1. Alport syndrome can result from mutations in three different genes. This is an example of:
   A. Allelic heterogeneity
   B. Cellular heterogeneity
   C. Clinical heterogeneity
   D. Locus heterogeneity
   E. Phenotypic heterogeneity
2. What is the probability that a third child born to this family would be affected with Alport syndrome?
   A. 10%
   B. 25%
   C. 50%
   D. 66%
   E. 75%
3. A 33-year-old male has a clinical diagnosis of Alport syndrome. He reports that his 60-year-old father has had recent episodes of hematuria. Which of the following sequence changes would BEST explain the phenotype in this family?
   A. *COL4A3* c.1452G>A (p.G484G)
   B. *COL4A3* c.1477G>A (p.G493S)

C. *COL4A5* c.1095G>A (p.G365G)
D. *COL4A5* c.2023G>A (p.G675S)
E. *COL4A5* c.5030G>A (p.R1677Q)

4. A.K.'s mother does not have features of Alport syndrome, but has the same mutation as her son who is affected. The clinical phenotype in females with X-linked Alport syndrome is MOST LIKELY modified by:
   A. Genomic variation
   B. Haplotype
   C. Non-penetrance
   D. Variable expressivity
   E. X inactivation
5. Which of the following mutation scanning methods would NOT be optimal for molecular diagnosis of Alport syndrome?
   A. Denaturing gradient gel electrophoresis (DGGE)
   B. Denaturing high performance liquid chromatography (DHPLC)
   C. Protein truncation test (PTT)
   D. Single strand conformational polymorphism (SSCP)
   E. Temperature gradient gel electrophoresis (TGGE)

Answers to Questions Embedded in the Text

Question 1: Draw a three-generation pedigree for this family (Fig. 2.2)

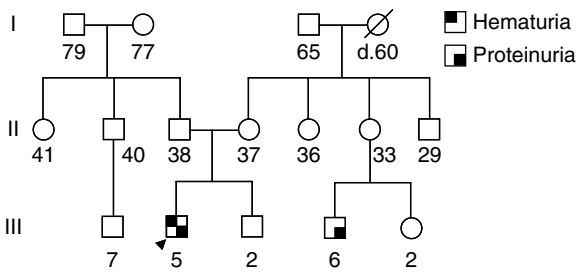

Fig. 2.2 Shown is a three-generation pedigree with the proband denoted by an arrow. Males are depicted with square symbols and females with circles. The ages of the individuals are shown. A slash through the symbol denotes a deceased individual with the age of death shown. Affected individuals are denoted by shaded blocks according to the key

Question 2: What is your differential diagnosis?

There are several causes of hematuria and proteinuria in children. The two most common causes of isolated hematuria are thin basement membrane nephropathy (TBMN) and Immunoglobulin A (IgA) nephropathy [9]. IgA nephropathy is the most common glomerulonephritis worldwide. It is an autoimmune disease in which deposition of the IgA antibody in the glomerulus results in inflammation. Because most cases of IgA nephropathy are sporadic, the diagnosis is unlikely in this family where the proband's cousin appears to be presenting with similar symptoms [10]. TBMN is associated with heterozygous mutations in *COL4A3* and *COL4A4* and may represent a mild form of Alport syndrome [4]. The presence of proteinuria in this family suggests the more severe Alport syndrome, since proteinuria is rarely observed in TBMN. Additionally, the family history appears to be consistent with an X-linked pattern of inheritance, thus implicating the X-linked *COL4A5* gene.

Question 3: What are the limitations and advantages of this approach?

A mutation scanning approach allows rapid analysis of all the exons and detection of known and novel mutations. For large genes, mutation scanning allows for a faster and less expensive method of mutation analysis than direct DNA sequencing. However, some mutation scanning approaches have limited sensitivity. HRMA has been reported to have >99% sensitivity for the detection of heterozygous variants in amplicons smaller than 500 bp [11]. HRMA has other advantages over other scanning methods: it is a closed-tube, one-step scanning method, and scanning is nondestructive so that positive amplicons can be directly analyzed by subsequent sequencing to identify the specific mutation. One limitation is that, since the sensitivity of HRMA is enhanced by the formation of heteroduplexes between wild-type and mutant DNA molecules, the sensitivity to detect homozygous or hemizygous variants is decreased. Mixing the DNA sample with an equal concentration of a normal control allows formation of heteroduplexes and increases the sensitivity of homozygote and hemizygote detection (see Fig. 2.1).

Another limitation is that mutation detection techniques such as HRMA and DNA sequencing will not detect large gene deletions or rearrangements. Sequencing analysis has a mutation detection rate of ~90% in patients with a typical presentation of Alport

syndrome and a family history consistent with X-linked inheritance [12]. Comprehensive molecular diagnosis requires additional dosage analysis for large structural rearrangements, particularly in affected females where the presence of a normal allele confounds interpretation of sequencing results.

Question 4: Does this result explain the patient's phenotype?

The reported *COL4A5* c.4946T>G (p.Leu1649Arg) mutation alters a conserved amino acid that is involved in intramolecular interactions within the non-collagenous (NC1) domain of the COL4A5 protein and is the molecular basis for the patient's renal symptoms. Mutations in the NC1 domain of COL4A5 affect the assembly of the collagen triple helical protomer. There is no clear genotype–phenotype correlation, but NC1 domain mutations may result in a more severe phenotype than glycine missense mutations, particularly those in the 5′ end of the gene [8]. *COL4A5* L1649R is a founder mutation that was initially reported at a high prevalence in a population from the western United States [1]. Affected males with this mutation have developed microscopic hematuria in childhood, but onset of renal failure was generally delayed until after 40 years of age and usually preceded hearing loss. Renal biopsy showed GBM alterations that are characteristic of Alport syndrome. A similar clinical course might be expected for this patient.

Answers to Multiple Choice Questions

1. *The correct answer is D.*

Locus heterogeneity refers to the fact that mutations in different genes (*COL4A3*, *COL4A4*, and *COL4A5)* result in the same phenotype of Alport syndrome. Choices A, C, and E are all true for Alport syndrome. Allelic heterogeneity refers to the fact that many different mutations within a given gene have been described in Alport syndrome. Clinical and phenotypic heterogeneity both refer to the presence of different symptoms and disease severity that can manifest in patients with Alport syndrome. Cellular heterogeneity refers to the presence of distinct cell types, such as within a tumor or cell culture.

2. *The correct answer is B.*

For this family, the disease-causing mutation appears to be non-penetrant in females, so only a boy inheriting the disease allele would be affected. Multiply the two independent variables: 1/2 (the probability of having a boy) × 1/2 (the probability that he will inherit the mutation) = 1/4 (25%).

3. *The correct answer is B.*

Choices A and C are benign synonymous single nucleotide polymorphisms. Choices B, D, and E are pathologic mutations that have been reported previously in association with Alport syndrome [12–14]. However, the inheritance pattern in this family from father to son excludes X-linkage, so a *COL4A5* mutation is very unlikely to be the disease-causing mutation in this family.

4. *The correct answer is E.*

X-inactivation is the mechanism by which one X-chromosome is randomly silenced in each cell of females, in order to equalize X-linked gene dosage between males and females. As a result, female carriers of X-linked diseases such as XLAS are usually unaffected or mildly affected except in cases of extremely skewed X-inactivation.

5. *The correct answer is C.*

PTT relies on identification of shortened protein fragments *in vitro*, so only nonsense or frameshift mutations can be detected by this method. Since these represent a small proportion of mutations in XLAS, PTT is not optimal for diagnosis of XLAS. The other choices are suitable mutation screening methods that can detect sequence variants based on different migration patterns of DNA molecules through an electrophoretic gel (DGGE, SSCP, and TGGE) or chromatography column (DHPLC).

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http://www.springer.com/978-3-642-19676-8

Diagnostic Molecular Pathology in Practice
A Case-Based Approach
Schrijver, I. (Ed.)
2011, XI, 339 p. 60 illus., 40 illus. in color., Softcover
ISBN: 978-3-642-19676-8