

An Exome Sequencing Study of 10 Families with IgA Nephropathy

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Abstract

Background: Immunoglobulin A nephropathy (IgAN) is a heterogeneous disorder with a strong genetic component. The advent of whole exome sequencing (WES) has accelerated the discovery of genetic risk factors underlying familial disorders. **Objectives:** We set out to test whether damaging variants in known kidney disease genes explain a proportion of IgAN cases recruited in Ireland. **Methods:** We performed WES in 10 Irish families with multiple affected members having kidney disease where at least one member had biopsy confirmed IgAN. Candidate variants were identified based on being shared between affected family members, minor allele frequency, function and predicted pathogenicity. Pathogenicity of variants was determined according to American College of Medical Genetics and Genomics guidelines. **Results:** We detected candidate variants in 3 of 10 families. We identified a likely pathogenic variant in *COL4A5* in one family and a variant of unknown significance (VUS) in *COL4A3* in another. Variants in *COL4A5* and *COL4A3* are

known to cause Alport syndrome. In the third family, we identified a VUS in *LMX1B*, a gene associated with Nail-patella syndrome. **Conclusions:** We identified a number of cases of familial IgAN where the families harbored variants in known kidney disease-related genes indicating that potentially a number of cases of familial IgAN are mistaken for other familial kidney disorders. However, the majority of families studied did not carry a candidate variant in a known kidney disease causing gene indicating that there may be >1 underlying genetic mechanism present in these families.

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Introduction

Immunoglobulin A nephropathy (IgAN) is a heterogeneous disorder defined by the presence of immunoglobulin A (IgA) deposits on immunofluorescence of kidney biopsies. It is the most common form of glomerulonephritis in the world and can occur de novo or associated with a multitude of different disease types [1].

G.L.C. and P.J.C. contributed equally to this work.

Table 1. Disease status numbers for familial IgAN families

Pedigree	Number of members affected	Number of generations affected	Number with ESRD	Number with biopsy proven IgAN	Mode of inheritance
F141	6	1	1	1	AD
F229	2	1	0	2	AR
F315	2	1	2	2	AD
F74	4	2	2	3	AD
F104	2	2	1	1	AR
F224	3	1	2	1	AR
F404	2	1	1	1	AD
F433	2	1	1	1	AD
F654	3	2	2	2	AD
F91	4	1	1	1	ADIP

AD, autosomal dominant; AR, autosomal recessive; ADIP, autosomal dominant with incomplete penetrance; IgAN, immunoglobulin A nephropathy; ESRD, end-stage renal disease.

The condition varies greatly in progression and severity [2] ranging from individuals developing end-stage renal disease to cases of asymptomatic mesangial IgA deposits seen post-mortem [3]. It affects people of all ages but most commonly in early adulthood [1]. More males are affected than females, with a reported ratio of approximately 2:1 [4, 5].

There are many pieces of evidence which suggest that genetic factors play a major role in disease occurrence and progression. These include many reports over the last number of decades, of clustering in families and different prevalence of the disease across ethnicities living in similar environments [6, 7]. Some of the reports of IgAN within families suggest a Mendelian pattern of inheritance [6], however, many of these families show incomplete penetrance, which fits with a multifactorial disease model and suggests that additional factors (either environmental or genetic) beyond a single causal mutational are needed to trigger the onset of the disease [6].

Despite >30 years of genetic analysis of IgAN, no clear causative gene has been identified. Linkage studies of families with IgAN have identified significantly associated loci including 3q24–23, 6q22–23 and 2q36 [8–10]. In spite of a number of linked loci, in the majority of these families, the causal gene has remained elusive.

In the last decade, a handful of next-generation sequencing studies have been carried out to identify candidate genes for IgAN. One such study carried out whole exome sequencing (WES) in 10 families with IgAN. They identified 6 candidate disease-causing variants in the genes *MYCT1*, *DEFA4*, *CARD8* and *ZNF543* [11]. Another WES study identified a novel deleterious variant in

the gene *SPRY2* that segregated with the disease in a large Sicilian family presenting with autosomal dominant IgAN. *SPRY2* is part of the MAPK/ERK pathway; however, the biological link between IgAN and MAPK/ERK pathway is yet to be explained [12].

A WES study by Cox et al. [13] examined rare variants segregating with IgAN in 16 families composed of 240 IgAN cases and 113 controls. They identified 23 genes with candidate disease-causing variants that were functionally related to a large immune-related network of pathways. They hypothesized that IgAN disease status may be influenced by a variety of mutations influencing one over-arching immune-related network [13].

To further expand upon these studies, we conducted WES on 10 Irish families who presented with multiple affected family members having severe kidney disease (stage 3 or above) in whom at least one member had biopsy confirmed IgAN (Table 1). In this study, we identified that a number of cases of familial IgAN are in fact likely mistaken for other familial kidney disorders. Our findings illustrate the role of molecular diagnosis in accurate disease classification.

Materials and Methods

Selection of Families

The Irish Kidney Gene Project is a national cross-sectional analysis of 1,809 patients who attended renal clinics and dialysis units in Ireland. Part of the project involved characterisation of family history among patients with kidney disease [14]. The Irish Kidney Gene Project and several other data sources were accessed to identify all cases for inclusion in this study of familial IgAN.

All patients in this study provided informed written consent. The study protocol and design were subject to appropriate Ethics Committee approval at Beaumont Hospital, Dublin, Ireland (research Ethics Committee reference: 12/75).

Families presenting with IgAN were identified through a clinical analysis carried out by Fennelly et al. [15]. Patients were selected on the basis that they had a clinical picture compatible with IgAN predominantly. Specifically patients with advanced liver disease that is associated with IgAN or patients with other disease that can show IgAN deposition such as Lupus or membranous nephropathy were excluded. In this study, 8,033 kidney biopsies performed at Beaumont Hospital over the period 1986–2016 were reviewed and identified 1,283 cases of IgAN. Of these cases, 23 (1.8%) were found to have a family history of kidney disease and were classed as familial IgAN. These 23 cases represented 14 different families. The families were then contacted and blood/saliva was collected on any available, consented family member by the staff at Beaumont Hospital. DNA from these patients was obtained at Beaumont Hospital from these collected blood or saliva samples and processed through the Rare Kidney Disease Biobank at St. James Hospital, Dublin, Ireland. We then chose families who had at least one family member with biopsy proven IgAN and one other family member with either biopsy-proven IgAN or end-stage renal disease as previously described [15] and selected families that had at least 2 affected members with DNA available. This left us with 10 families for the exome sequencing analysis. All unaffected individuals that had gene sequencing were labelled unaffected on the basis of negative dipstick for significant blood or proteinuria. All families recruited were Irish and were reported as non-consanguineous.

Ten families were selected for exome sequencing, labelled F104, F141, F229, F315, F74, F404, F433, F654, F224, and F91. In family F141, we sequenced 3 affected members. In family F104, we sequenced one affected member and in the remaining 8 families, we sequenced 2 affected members (F229, F315, F74, F404, F433, F654, F224, and F91). Families were selected for exome sequencing on the basis of meeting the aforementioned phenotype criteria and also having sufficient DNA to carry out exome sequencing and test for segregation, as required.

WES Analysis

We exome sequenced the 20 individuals from 10 families to 100X coverage using either an Ion Proton platform ($n = 6$, families = F229, F315 and F91) or an Illumina HiSeq platform ($n = 14$, families = F141, F74, F104, F224, F404, F433 and F654). Six individuals were sequenced using the SureSelect XT Human All Exon V6 + UTRs Kit (members from F141, F74 and F104), 8 were sequenced using the SureSelect XT Human All Exon V6 Kit (members from F224, F404, F433 and F654), 2 were sequenced with the Ion TargetSeq Exome (both individuals in F91) and the remaining 4 were sequenced using the Ion Ampliseq exome RDY kit (families F229 and F315).

Annotating Variants

Following alignment and quality control of exome data (online suppl. Methods; for all online suppl. material, see www.karger.com/doi/10.1159/000503564), VCF files were annotated using Annovar [16]. Candidate variants were defined as variants that were shared between affected family members, functional, rare and predicted deleterious (online suppl. Methods).

Candidate variants and where relevant, the associated genes were cross-referenced with the Online Mendelian Inheritance in Man catalog, Clinvar (version 20150330), gnomad, missense tolerance ratio Gene Viewer and Residual Variation Intolerance Score [17–21].

We incorporated clinical data using Online Mendelian Inheritance in Man and Clinvar to see if any of the candidate variants were thought to be involved in kidney disease or had been previously been reported as pathogenic. We then re-examined the pedigree information to select variants that fit the disease model. Finally, we used the American College of Medical Genetics and Genomics (ACMG) guidelines to annotate whether or not the variant was considered pathogenic, likely pathogenic, uncertain significance, likely benign or benign [22].

We used Sanger sequencing to confirm candidate variants and to test for segregation (online suppl. Methods).

Searching for Variants Across Families

Families in which we did not identify a candidate variant in a gene previously linked with renal disease were assessed for candidate variants found in genes shared across these families. Genes with a candidate variant found in >1 family were selected. Artefacts of sequencing were identified through examination of the bam files using the Integrative Genomics Viewer [23].

Results

We performed WES on 20 individuals across 10 families (Table 1). The average age in the affected individuals ($n = 30$) across these 10 families was 41 (SD 15.8 years), with 22 males and 8 females (online suppl. Table 1 for further details on affected family members). The average age in the unaffected individuals ($n = 26$) across these 10 families was 58 (SD 11.6 years), with 8 males and 17 females. In 7 families (F224, F91, F404, F433, F654, F229 and F315), we did not identify candidate variants in genes known to cause kidney disease, or that were found across multiple families in biologically relevant genes (online suppl. Table 2 for list of qualifying variants found in these families). In 3 families (F141, F74 and F104), we identified candidate mutations that were found in genes known to cause kidney disease (Table 2).

Exome Sequencing: Family 141

Family 141 (F141) is a multi-generational family with multiple affected members presenting with blood and proteinuria. In one member a kidney biopsy confirmed IgA deposition in the mesangium. There was clear male to male transmission suggesting an autosomal dominant mode of inheritance (Fig. 1). The proband (IV.1) in this family was diagnosed (via biopsy) with IgAN and mild arteriosclerosis/arteriolar sclerosis (Fig. 2). The proband's father (III.6) was diagnosed with focal prolifera-

Table 2. Candidate variants found in 3 Irish families with IgAN

Family	rsID	Gene	Chr	BP	Ref./Alt	1000G	ExAC	ESP	SIFT	Polyphen2	CADD
F74	rs373336352*	<i>LMX1B</i>	9	129458213	G/A	NA	NA	0.0001	0.49 (T)	0.99 (D)	10.5
F141	rs104886228**	<i>COL4A5</i>	X	107908790	G/A	NA	NA	NA	0 (D)	1 (D)	21.7
F104	rs200302125***	<i>COL4A3</i>	2	228172594	T/C	0.0012	0.003	0.003	0 (D)	1 (D)	15.76

* The highest MAF seen for rs373336352 was in the ESP European subpopulation (MAF = 0.0001).

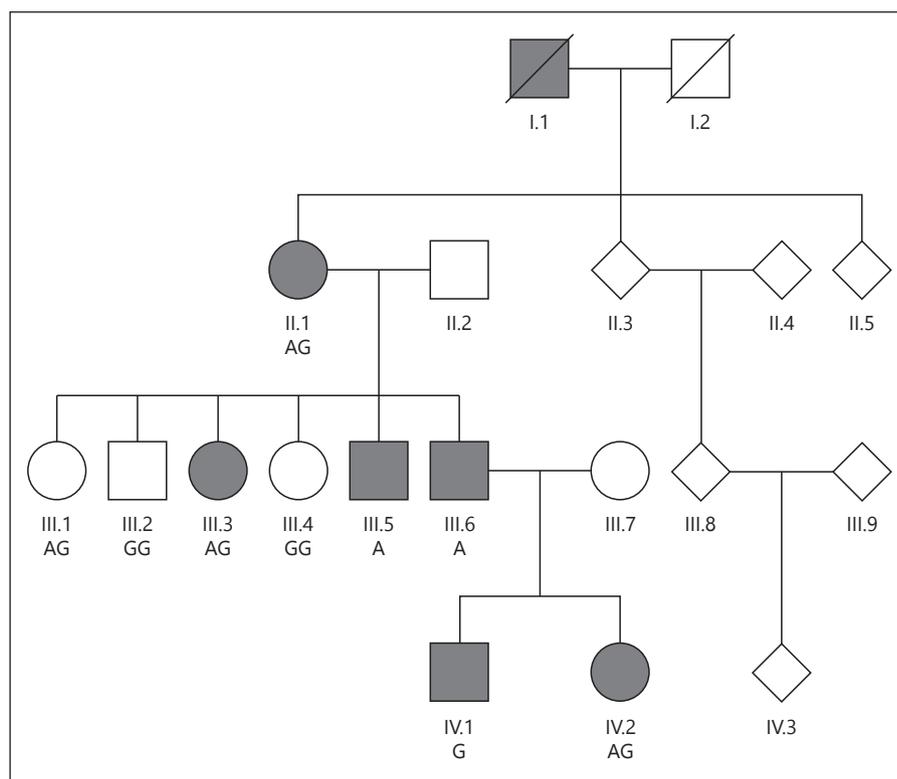
** rs104886228 was not seen in any of the subpopulations in ExAC, 1000G or ESP.

*** The highest MAF seen for rs200302125 was in the ExAC non-Finnish European population (MAF = 0.0046).

All the variants in the above table were nonsynonymous SNVs found in exonic regions.

rsID, SNP identifier; Chr, chromosome; BP, base pair position (hg19 coordinates); 1000G, minor allele frequency in across all populations in the 1,000 Genomes project using the August 18, 2015 Annovar release; ExAC, Minor allele frequency across all populations in the Exome Aggregation Consortium (ExAC; exac03 version); ESP, Minor allele frequency of variant in the Exome Sequencing Project (eps6500siv2 ALL release); Polyphen2, Polyphen2 HDIV score (prediction); SIFT, SIFT score (prediction); T, tolerated; D, damaging; CADD, CADD phred score; *LMX1B*, lim homeobox transcription factor 1, beta; *COL4A5*, collagen, type IV, alpha-5; *COL4A3*, collagen, type IV, alpha-3; IgAN, immunoglobulin A nephropathy.

Fig. 1. Pedigree of family F141. Circles indicate females, squares indicate males, diamond indicates uncertain sex, arrow indicates proband, diagonal line through a shape indicates a deceased individual, filled shapes indicates affected status, unfilled shapes indicate an unaffected individual. Individuals with A or G letter under their shape indicates their genotype at the *COL4A5* variant. The proband (IV.1), father (III.6) and uncle (III.5) underwent biopsies.

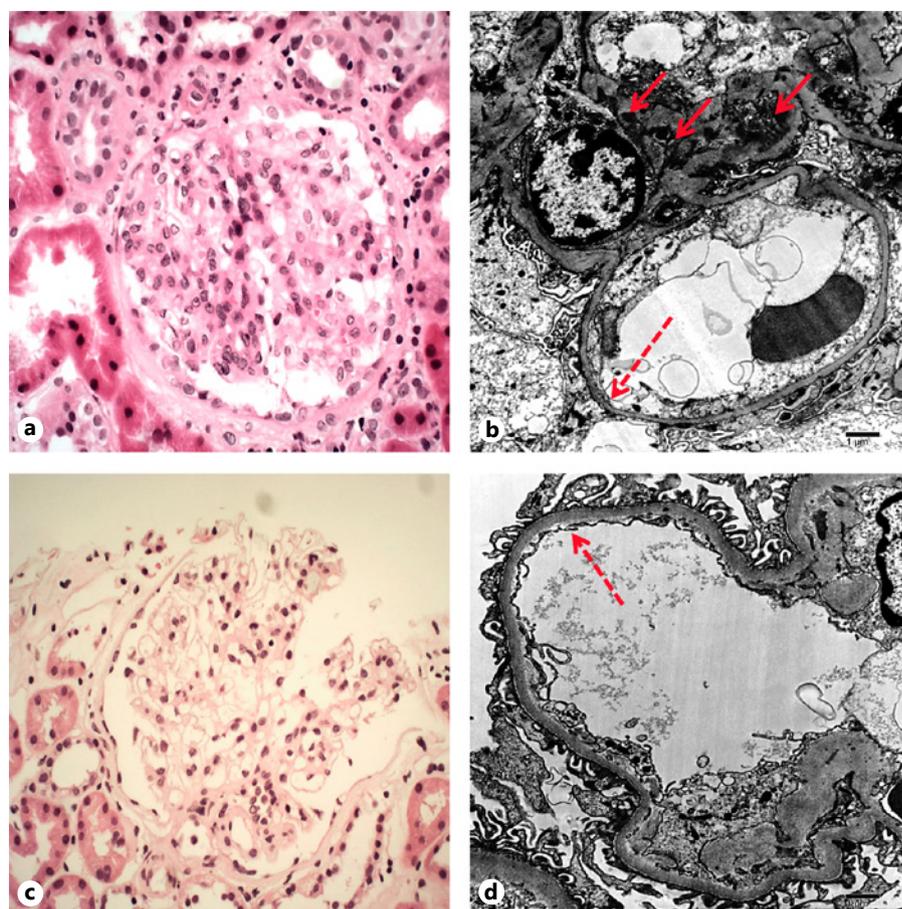


tive glomerular nephropathy, thin glomerular basement membrane and mild arteriolar sclerosis. The proband's grandmother (II.1) was reported to have "scarred kidneys" but the reason for this scarring was not reported. The proband's sister (IV.2) had signs of early stage chronic kidney disease with both blood and proteins present in the urine. The proband's paternal uncle (III.5) was also found to have kidney disease with urinalysis showing presence of protein and haemolysed blood and upon biopsy showed signs of thin basement membrane nephropathy (Fig. 2). The proband's aunt (III.3) also presented

with 2+ blood in the urinalysis. A cousin (IV.3) was reported as affected, but exact details on this individual are unknown.

Three of the affected individuals were selected for WES (Fig. 1) – the proband (IV.1), the sister (IV.2) and the affected uncle (III.5). In F141, we identified a likely pathogenic variant (according to ACMG standards) in the gene *COL4A5*, a gene known to cause X-linked Alport syndrome [24]. This variant was present in IV2 and III5 but not in the proband. This variant, rs104886228: G>A, was found on chromosome X in the 38th exon of *COL4A5*.

Fig. 2. Light and electron micrographs showing appearances seen in IgAN and Thin basement membrane nephropathy of family 141. **a** Hematoxylin and Eosin stained light micrograph (20×) showing diffuse mesangial matrix expansion and hypercellularity from kidney cortex of individual IV1 from F141. **b** Electron micrograph (5,000×) showing electron dense deposits in the mesangium (solid arrows) and a thin basement membrane on the glomerular capillary loop (dashed arrow) from individual IV1. **c** Hematoxylin and Eosin stained light micrograph (20×) showing a normal glomerulus in individual III.5. **d** Electron micrograph (10,000×) showing a thin glomerular basement membrane in individual III.5 (dashed arrow). No electron dense deposits were found in the mesangium of III.5. In individual IV1, immunofluorescence showed global and diffuse granular mesangial positivity for IgA in all glomeruli (data not shown), while no evidence of immune complex deposition was seen in individual III.5. The findings in the above images, taken together with the immunofluorescence results, demonstrate features typical of IgAN and thin basement membrane nephropathy for IV1 and TBMN for III.5.



Color version available online

This nucleotide change was a nonsynonymous single nucleotide variation (SNV) predicted to result in an amino acid change (glycine to serine) at position 1,143 of the *COL4A5* protein p.(Gly1143Ser). We interpreted this variant according to ACMG criteria as “likely pathogenic” (Table 3).

We went on to genotype the variant in 9 family members (6 affected and 3 unaffected) to test for segregation. The “likely pathogenic” (“A”) allele was found in to segregate with affected status along the proband’s father’s side of the family (Fig. 1, online suppl. Fig. 1). As this variant is on the X chromosome, the father cannot pass the mutation to the proband indicating that there may be 2 very similar diseases segregating in 1 family. Notably, there was one healthy female carrier (III.1) who was found to have a clear urinalysis (urinalysis carried out at age 44). Due to potential X-inactivation [25, 26] and the segregation pattern of the variant, we did not consider cosegregation data as evidence towards ACMG. This pedigree appears to demonstrate sporadic IgAN within a family with Alport mutations. This case demonstrates the

difficulty of studying familial kidney disease where 2 kidney disease patterns occur within the 1 family or where a family member is a phenocopy with a different disease.

Exome Sequencing: Family 104

Family 104 (F104) presented with 2 affected brothers with a recessive inheritance pattern (Fig. 3). The first affected brother (II.1) presented with hematuria and proteinuria at the age of 29 and was diagnosed with IgAN upon biopsy. He progressed to end-stage kidney disease undergoing transplant approximately 6 years after his IgAN diagnosis. The second brother (II.3) was also diagnosed with IgAN. No other family members reported kidney disease.

In F104, a variant of unknown significance (VUS, according to ACMG guidelines), rs200302125: T>C, was identified in the non-collagenous domain of *COL4A3* (Table 2, Fig. 3). Here, a nonsynonymous SNV was predicted to result in the replacement of a leucine with a proline at amino acid position 1474 p.(Leu1474Pro). *COL4A3* is associated with autosomal dominant and recessive

Table 3. Criteria met by *COL4A5* variant found in F141

ACMG criteria met	Evidence
PM1 – Located in a mutational hot spot and/or critical and well-established functional domain (e.g., the active site of an enzyme) without benign variation	Variant was found in the in the collagenous domain of the $\alpha 5$ (IV) chain. Glycine substitutions in this region are known to have deleterious effects on the function of the protein [35].
PM2 – Absent from controls (or at an extremely low frequency if recessive) in Exome Sequencing Project, 1,000 Genomes Project, or Exome Aggregation Consortium	Variant not found in gnomad, 1000G_ALL, ExAC_ALL or ESP6500siv2_ALL
PM5 – Novel missense change at an amino acid residue where a different missense change determined to be pathogenic has been seen before	Variant was at the same codon but causing a different amino acid (glycine to aspartate) change that had previously been found in a family with Alport syndrome and is reported as pathogenic in OMIM [34]. This variant was located in the collagenous domain of the $\alpha 5$ (IV) chain and was found interfere with the maintenance of the triple helical formation of the collagen molecule which leads to increased permeability and weakening of the glomerular basement membrane
PP2 – Missense variant in a gene that has a low rate of benign missense variation and in which missense variants are a common mechanism of disease	<i>COL4A5</i> has an RVIS score = 6.3%, meaning this gene is amongst the top 6.3% most intolerant genes in the human genome (based upon the amount of common variation found in that gene) [18].
PP3 – Multiple lines of computational evidence support a deleterious effect on the gene or gene product (conservation, evolutionary, splicing impact, etc.)	Variant was predicted damaging (D) by SIFT_pred, Polyphen2_HDIV_pred and MutationTaster_pred. CADD_phred score of 21.7
PP5 – Reputable source recently reports variant as pathogenic, but the evidence is not available to the laboratory to perform an independent evaluation	Previous studies identified variant as pathogenic in families with Alport syndrome, however this variant not reported in OMIM [35, 49].

The above table lays out the evidence for calling the *COL4A5* variant found in F141 as likely pathogenic according to ACMG guidelines.

PM, moderate pathogenic criterion; PP, supporting pathogenic criterion; ACMG, American College of Medical Genetics and Genomics; OMIM, Online Mendelian Inheritance in Man.

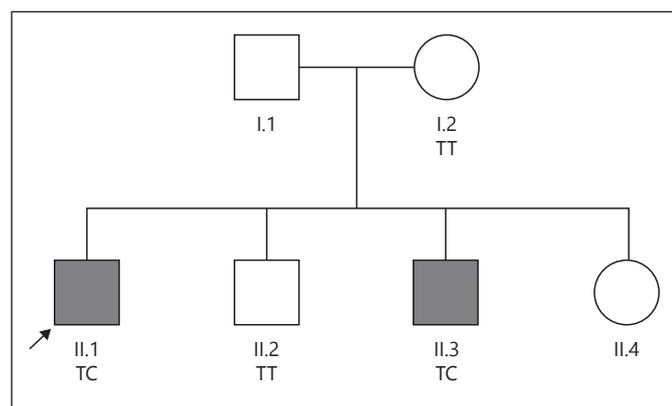


Fig. 3. Pedigree of family F104. Circles indicate females, squares indicate males, diamond indicates uncertain sex, arrow indicates proband, filled shapes indicates affected status, unfilled shaped indicate an unaffected individual. Individuals with T or C letter under their shape indicates their genotype at the *COL4A3* variant. Individual II.1 underwent biopsy.

forms of Alport syndrome as well as autosomal dominant benign familial hematuria [27–32].

The minor allele frequency was high for a disease-causing heterozygote variant (0.12% MAF in 1000G_All and 0.2% in gnomad). Several computational tools predicted the variant to be damaging (SIFT_pred = D, Polyphen2_HDIV_pred = D, MutationTaster_pred = D, CADD_phred = 15.76). The variant met a number of criteria for ACMG, as laid out in Table 4. However, the evidence was not sufficient to call this variant as pathogenic, and so at the time of writing, this variant comes under the VUS category.

Exome Sequencing: Family 74

The third family, family 74 (F74), presented with an autosomal dominant inheritance pattern with 4 affected individuals across 2 generations (Fig. 4). The proband (II.11) presented with biopsy confirmed IgAN and diabetic nephropathy, which progressed to end-stage kidney disease and required the patient to undergo transplant.

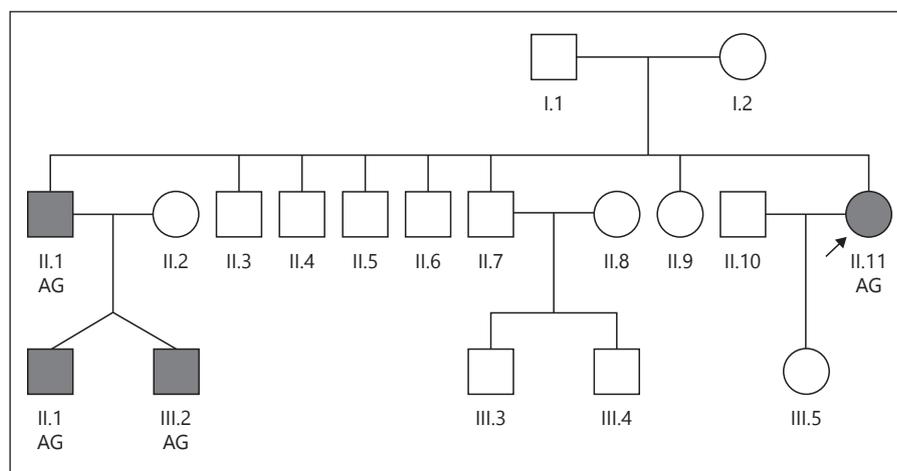
Table 4. Criteria met by *COL4A3* variant found in F104

ACMG criteria met	Evidence
PP1 – cosegregation with disease in multiple affected family members in a gene definitively known to cause the disease	Variant segregated with the disease in 2 unaffected and 2 affected family members (online suppl. Fig. 2)
PP3 – multiple lines of computational evidence support a deleterious effect on the gene or gene product (conservation, evolutionary, splicing impact, etc.)	Several computational tools predicted the variant to be damaging (SIFT_pred = D, Polyphen2_HDIV_pred = D, MutationTaster_pred = D, CADD_phred = 15.76)
PP5 – reputable source recently reports variant as pathogenic, but the evidence is not available to the laboratory to perform an independent evaluation	A recent study reported the same variant in a family with (likely mistaken) familial focal segmental glomerulosclerosis [37].

The above table lays out the evidence for calling the *COL4A3* variant found in F104 as uncertain significance according to ACMG guidelines.

PP, supporting pathogenic criterion; ACMG, American College of Medical Genetics and Genomics.

Fig. 4. Pedigree of family F74. Circles indicate females, squares indicate males, diamond indicates uncertain sex, arrow indicates proband, filled shapes indicates affected status, unfilled shaped indicate an unaffected individual. Individuals with A or G letter under their shape indicates their genotype at the *LMX1B* variant. Individuals II.1, III.1 and II.11 underwent biopsy.



The proband's nephew (III.1) presented with biopsy confirmed IgAN and mild arteriosclerosis/arteriolar sclerosis (Fig. 5). The second nephew (III.2) of the proband self-reported as healthy, however, urinalysis showed haemolysed blood. The proband's brother (II.1) presented with CKD and on renal biopsy IgA deposits were found in the glomerular mesangium (Fig. 5).

Two individuals in F74 were selected for WES (II.1 and III.1). We identified a candidate variant, rs373336352: G>A, in the gene *LMX1B*, a gene associated with Nail-patella syndrome (Table 2, Fig. 4) [33]. This variant, was a nonsynonymous SNV which was predicted to cause an amino acid change from glycine to serine, p.(Gly339Ser). Although this variant was also found to segregate with the disease in affected family members, it had mixed results for computational evidence supporting a deleterious effect on the gene or gene product (CADD Phred score of

10.5, Polyphen2_HDIV_pred = D, MutationTaster = D, SIFT_pred = T) and had a higher MAF than would be expected for a pathogenic variant. As such, it was classified as VUS (Table 5). It was not possible to obtain DNA samples for unaffected family members in F74 and therefore only affected family members were sequenced.

Discussion

We analysed exome sequence data from 10 Irish families presenting with IgAN, filtering for rare, functional, exonic or splicing, predicted damaging variants that were shared between affected members of the family. We detected an ACMG-satisfying disease causing mutation in one of the 10 families, with VUS found in kidney-disease-related genes in another 2 families.

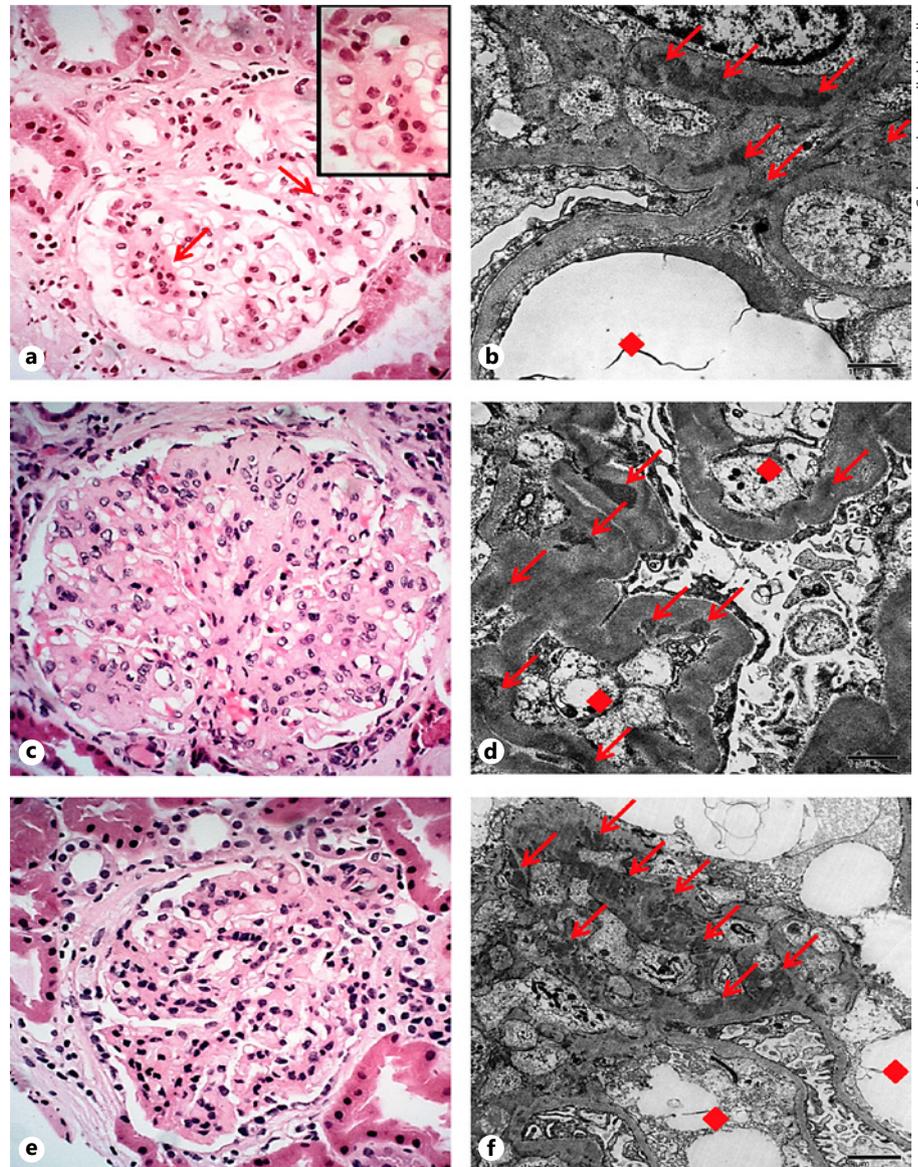


Fig. 5. Light and electron micrographs showing appearances seen in IgAN of family 74. **a** Hematoxylin and Eosin stained light micrograph (20 \times) showing areas of mesangial hypercellularity and mesangial matrix expansion (arrows) from kidney cortex of individual II.11. The inset shows one of these areas at higher magnification (40 \times). **c** Hematoxylin and Eosin stained light micrographs (20 \times) showing diffuse mesangial hypercellularity and expansion from kidney cortex of individuals II.1 and III.1, respectively. **b, d, e** Electron micrographs (15,000 \times) showing electron dense deposits in the mesangium (arrows) and also capillary loops (**b**). The diamonds show the glomerular capillary loop spaces. Immunofluorescence for all patients demonstrated global and diffuse granular mesangial positivity for IgA (data not shown). The findings in the above images, taken together with the immunofluorescence results, demonstrate the features typical of IgAN.

IgAN is the most common form of glomerulonephritis worldwide and likely has multiple different forms, from monogenic to multifactorial. Despite strong evidence from familial segregation, to date a clearly monogenic form has yet to be fully characterised. Given the relative frequency of IgAN, it is also clear that IgA deposits may be present on kidney biopsies with a second clear renal pathological diagnosis. This points to the difficulty in analysing kidney pathology where IgA deposition may be present but other disease mechanisms are the predominant lesion affecting kidney structure. In the families presented in this study, there was clear evidence of IgA deposition thus making the criteria for a diagnosis of IgAN in extended families with advanced kidney disease.

In our analysis, we did not identify any potentially causal qualifying variants in 7 of the families. We made a clear molecular diagnosis in one case and in 2 further cases, we identified variants of uncertain significance. In family F141, a likely pathogenic mutation was found in the *COL4A5* gene (notably, this variant did not meet the criteria originally set out in this study as it was not present in the proband). Mutations in this gene are commonly associated with Alport syndrome. This variant and a mutation resulting in a different amino acid change at the same codon had previously been identified as pathogenic in families with Alport syndrome [34, 35]. In F141, the father's biopsy report diagnosis was focal proliferative glomerular nephropathy with mild arteriolar sclerosis

Table 5. Criteria met by *LMX1B* variant found in F74

ACMG criteria met	Evidence
PP1 – cosegregation with disease in multiple affected family members in a gene definitively known to cause the disease	The variant segregated with all 4 affected family members (online suppl. Fig. 3)
PP2 – Missense variant in a gene that has a low rate of benign missense variation and in which missense variants are a common mechanism of disease	<i>LMX1B</i> has RVIS score of 11.06% and a number of missense variants reported as pathogenic in OMIM
BS1 Allele frequency is greater than expected for the disorder	Variant was not found in 1,000 genomes, EXAC or gnomad databases but had a frequency of 0.0001 in the NHLBI-ESP project with 6,500 exomes. The variant was found in one individual in this database

The above table lays out the evidence for calling the *LMX1B* variant found in F74 as uncertain significance according to ACMG guidelines.

PP, supporting pathogenic criterion; BS, strong benign criterion; OMIM, Online Mendelian Inheritance in Man; ACMG, American College of Medical Genetics and Genomics.

and the uncles' biopsy showed thin basement membrane nephropathy (Fig. 2). In comparison, the proband's biopsy report showed IgAN.

This finding indicates that the father (and associated carriers of the *COL4A5* variant) had a similar, yet different, cause of kidney disease compared to the proband, who has clear features of IgAN and does not carry the *COL4A5* variant. Deafness, a clinical phenotype often found in individuals with Alport syndrome, was not found in this family [36]. This highlights the range and diversity in the phenotypes associated with type 4 collagen mutations. The proband in this family had clear evidence of IgAN with multiple affected family members with overt kidney disease which was likely resulting from collagen mediated disease. This family illustrates the difficulty in diagnosing familial kidney disease as there was clear evidence of 2 distinct pathogenic processes coexisting within the 1 family (IgAN and collagen basement membrane disease).

In family F104, we identified a VUS in *COL4A3*. This gene has previously been shown to cause autosomal recessive and dominant Alport syndrome as well as autosomal dominant benign familial haematuria [28]. This variant was recently reported in a family diagnosed with focal segmental glomerular sclerosis indicating that the spectrum of disease caused by mutations in *COL4A3* may be wider than previously thought [37]. However, the variant had a minor allele frequency that is higher than what we would expect for a causal heterozygous pathogenic variant. That being said, kidney disease is relatively common in any population. According to a 2010 study, the age-standardized global prevalence of CKD in adults (mini-

num age of 20) is 10.4% in men and 11.8% in women [38]. Therefore, it is possible that variants with relatively high minor allele frequencies, may in fact be causal, or contributing risk. Also, kidney disease has an extremely broad age of onset, becoming more common as age increases [39]. This means that variants causing kidney disease may not be under the same selective pressure as variants causing disease with earlier ages of onset as the mean age for end stage kidney disease in patients with IgAN is past the average age for child bearing (mean age recently reported as 42.8 [40]). Therefore, variants causing the disease could be at relatively high frequencies in the general population. However, without further evidence we cannot class the mutation found in this family as pathogenic and so whether this variant is causal or an incidental finding has yet to be determined.

In the third family, F74, we identified a variant in the gene *LMX1B*, known to cause autosomal dominant Nail-patella syndrome. This syndrome is characterised by dysplastic nails, absent or hypoplastic patellae and, in a number of cases, nephropathy resembling glomerulonephritis. *LMX1B* binds the enhancers of *COL4A4* [41]. In mice with *LMX1B* knock-out mutations (-/-), there is a strong decrease in the alpha-3 and -4 chains of type IV collagen [41]. Similar to that of Alport syndrome, the dysregulation of the alpha-3 and -4 chains of type IV collagen results in glomerulopathy. It is thought that haploinsufficiency is the pathogenic mechanism of Nail-patella syndrome, with the disease following an autosomal dominant inheritance pattern [33]. A study examining individuals with nail patella-like renal disease reported a number of families who carried a mutation in the home-

odomain of *LMX1B* indicating that individuals with mutations in this gene do not always present with the full spectrum of Nail-patella syndrome phenotypes and can cause renal specific disease [42]. However, it should be kept in mind that the variant in *LMX1B* was found at a higher frequency than expected, was not found in the homeodomain region [43] and had conflicting computational evidence for pathogenicity and so whether this variant is causal or an incidental finding remains uncertain. Investigations of the prevalence of variants of unknown significance in genes known to cause kidney disease are warranted to help us understand the likelihood of these variants occurring.

In the remaining 7 families, there was just one gene, *PIK3C2B*, that contained qualifying variants in >1 family (online suppl. Table 2). We identified 1 family (F91) that carried a frameshift deletion and 1 family (F433) containing a nonsynonymous variant in *PIK3C2B*. This gene has no known links to kidney disease and according to Residual Variation Intolerance Score, is in the 77.93% most intolerant genes (i.e., very tolerant to mutations) and so this may be an incidental finding or potentially is a risk factor as opposed to a causal variant – adding to an overall polygenic load. We did not identify any other genes with qualifying variants that were shared across these families. This raises a number of potential scenarios. Potentially, the disease in these families is caused by non-genetic (or at least not exclusively genetic) factors. IgAN is correlated with a number of environmental factors in particular infections. For example, HIV, staphylococcus mucosal infections, Chlamydia, malaria and Lyme disease have all been associated with the occurrence of IgAN [44–47].

Also, some forms IgAN may be polygenic. As IgAN is a relatively heterogeneous disorder, it is possible that different families have different underlying genetic causes in genes yet to be linked to kidney disease. Notably, a number of the families carried variants in genes (*TRAF2* and *NFKB2*, online suppl. Table 2) associated with TRAF6 mediated NF- κ B activation. TRAF6 mediated NF- κ B activation is involved in the production of galactose-deficient IgA1, which is needed for the formation of immune complexes found in individuals with IgAN [48]. Potentially, variants in the pathway and other pathways associated with the production of galactose-deficient IgA1 may be risk factors for the development of IgAN. Further analysis of larger cohorts of families presenting with IgAN will be needed to decipher this important question.

This study had a number of limitations. First, the absence of a control group of Irish ancestry would have al-

lowed us to better interpret the candidate variants presented in this study in the context of the given population. Further work is required to decipher the frequency of these variants in the Irish population. Another limitation of this study was that different sequencing platforms (Ion Torrent and Illumina) were used across the families. The resulting variants were refined using visual inspection with Integrative Genomics Viewer and followed up with Sanger sequencing to reduce the number of potential artefacts. However, the use of different sequencing platforms may have resulted in false negatives, where, for example, variants could be identified in 1 family was not observed in other families due to the differences in sequencing methodology. Also, due to the relatively small pool of families ($n = 10$), we were underpowered to carry out polygenic or burden analyses.

Our results confirm the heterogeneous nature of IgAN and provide evidence that while a proportion of families who present with IgAN may carry a disease causing variant in a known kidney disease-related gene, the majority do not. With the continued discovery of disease genes, it is possible that families with a currently unknown genetic cause for their disorder may receive a genetic diagnosis in the near future. Further analysis of large, carefully phenotyped families with IgAN should provide insights into disease mechanisms of IgAN.

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Statement of Ethics

Subjects have given their written informed consent. The study protocol has been approved by the Beaumont Hospital Ethics committee (research Ethics Committee reference: 12/75).

Disclosure Statement

The authors declare that they have no conflicts of interest to disclose.

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Author Contributions

N.K.F., C.K., D.M.C., and S.L.M. recruited patients, compiled pedigrees, gathered patient phenotype data and collected blood/saliva samples; C.P.S., G.L.C., and P.J.C. designed the analyses;

C.P.S. carried out DNA sample preparation and data analysis. G.L.C. and P.J.C. supervised the study; C.P.S. wrote the manuscript with consultation and guidance from G.L.C., P.J.C., and N.K.F.; N.K.F., A.M.D., and B.D. imaged and analysed biopsies. All authors reviewed and gave input to the final manuscript.

References

- 1 D'Amico G. The commonest glomerulonephritis in the world: IgA nephropathy. *Q J Med.* 1987 Sep;64(245):709–27.
- 2 Hsu SI, Feehally J. The molecular basis of IgA nephropathy. In: Mount DB, Pollak MR, editors. *Molecular and genetic basis of renal disease: a companion to Brenner & Rector's the kidney.* Philadelphia: Saunders/Elsevier; 2008. pp. 481–97.
- 3 Waldherr R, Rambauser M, Duncker WD, Ritz E. Frequency of mesangial IgA deposits in a non-selected autopsy series. *Nephrol Dial Transplant.* 1989;4(11):943–6.
- 4 Wyatt RJ, Julian BA. IgA nephropathy. *N Engl J Med.* 2013 Jun;368(25):2402–14.
- 5 Schena FP, Cerullo G, Rossini M, Lanzilotta SG, D'Altri C, Manno C. Increased risk of end-stage renal disease in familial IgA nephropathy. *J Am Soc Nephrol.* 2002 Feb;13(2):453–60.
- 6 Kiryluk K, Julian BA, Wyatt RJ, Scolari F, Zhang H, Novak J, et al. Genetic studies of IgA nephropathy: past, present, and future. *Pediatr Nephrol.* 2010 Nov;25(11):2257–68.
- 7 Hall YN, Fuentes EF, Chertow GM, Olson JL. Race/ethnicity and disease severity in IgA nephropathy. *BMC Nephrol.* 2004 Sep;5(1):10.
- 8 Gharavi AG, Yan Y, Scolari F, Schena FP, Frasca GM, Ghiggeri GM, et al. IgA nephropathy, the most common cause of glomerulonephritis, is linked to 6q22–23. *Nat Genet.* 2000 Nov;26(3):354–7.
- 9 Bisceglia L, Cerullo G, Forabosco P, Torres DD, Scolari F, Di Perna M, et al.; European IgAN Consortium. Genetic heterogeneity in Italian families with IgA nephropathy: suggestive linkage for two novel IgA nephropathy loci. *Am J Hum Genet.* 2006 Dec;79(6):1130–4.
- 10 Paterson AD, Liu XQ, Wang K, Magistroni R, Song X, Kappel J, et al. Genome-wide linkage scan of a large family with IgA nephropathy localizes a novel susceptibility locus to chromosome 2q36. *J Am Soc Nephrol.* 2007 Aug;18(8):2408–15.
- 11 Liu R, Hu B, Li Q, Jing X, Zhong C, Chang Y, et al. Novel genes and variants associated with IgA nephropathy by co-segregating with the disease phenotypes in 10 IgAN families. *Gene.* 2015 Oct;571(1):43–51.
- 12 Milillo A, La Carpia F, Costanzi S, D'Urbano V, Martini M, Lanuti P, et al. A SPRY2 mutation leading to MAPK/ERK pathway inhibition is associated with an autosomal dominant form of IgA nephropathy. *Eur J Hum Genet.* 2015 Dec;23(12):1673–8.
- 13 Cox SN, Pesce F, El-Sayed Moustafa JS, Salustio F, Serino G, Kkhoufou C, et al.; European IgAN Consortium. Multiple rare genetic variants co-segregating with familial IgA nephropathy all act within a single immune-related network. *J Intern Med.* 2017 Feb;281(2):189–205.
- 14 Connaughton DM, Bukhari S, Conlon P, Cassidy E, O'Toole M, Mohamad M, et al. The Irish Kidney Gene Project—Prevalence of Family History in Patients with Kidney Disease in Ireland. *Nephron.* 2015;130(4):293–301.
- 15 Fennelly NK, Kennedy C, Jenkinson AC, Connaughton DM, Stapleton C, Dorman AM, et al. Clinical Heterogeneity in Familial IgA Nephropathy. *Nephron.* 2018;139(1):63–9.
- 16 Wang K, Li M, Hakonarson H. ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. *Nucleic Acids Res.* 2010 Sep;38(16):e164.
- 17 Hamosh A, Scott AF, Amberger J, Valle D, McKusick VA. Online Mendelian Inheritance in Man (OMIM). *Hum Mutat.* 2000;15(1):57–61.
- 18 Petrovski S, Wang Q, Heinzen EL, Allen AS, Goldstein DB. Genic intolerance to functional variation and the interpretation of personal genomes. *PLoS Genet.* 2013;9(8):e1003709.
- 19 Traynelis J, Silk M, Wang Q, Berkovic SF, Liu L, Ascher DB, et al. Optimizing genomic medicine in epilepsy through a gene-customized approach to missense variant interpretation. *Genome Res.* 2017 Oct;27(10):1715–29.
- 20 Lek M, Karczewski KJ, Minikel EV, Samocha KE, Banks E, Fennell T, et al.; Exome Aggregation Consortium. Analysis of protein-coding genetic variation in 60,706 humans. *Nature.* 2016 Aug;536(7616):285–91.
- 21 Landrum MJ, Lee JM, Benson M, Brown GR, Chao C, Chitipiralla S, et al. ClinVar: improving access to variant interpretations and supporting evidence. *Nucleic Acids Res.* 2018 Jan;46(D1):D1062–7.
- 22 Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, et al.; ACMG Laboratory Quality Assurance Committee. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med.* 2015 May;17(5):405–24.
- 23 Robinson JT, Thorvaldsdóttir H, Wenger AM, Zehir A, Mesirov JP. Variant Review with the Integrative Genomics Viewer. *Cancer Res.* 2017 Nov;77(21):e31–4.
- 24 Lemmink HH, Schröder CH, Monnens LA, Smeets HJ. The clinical spectrum of type IV collagen mutations. *Hum Mutat.* 1997;9(6):477–99.
- 25 Nakanishi K, Iijima K, Kuroda N, Inoue Y, Sado Y, Nakamura H, et al. Comparison of alpha5(IV) collagen chain expression in skin with disease severity in women with X-linked Alport syndrome. *J Am Soc Nephrol.* 1998 Aug;9(8):1433–40.
- 26 Rheault MN, Kren SM, Hartich LA, Wall M, Thomas W, Mesa HA, et al. X-inactivation modifies disease severity in female carriers of murine X-linked Alport syndrome. *Nephrol Dial Transplant.* 2010 Mar;25(3):764–9.
- 27 Storey H, Savige J, Sivakumar V, Abbs S, Flinter FA. COL4A3/COL4A4 mutations and features in individuals with autosomal recessive Alport syndrome. *J Am Soc Nephrol.* 2013 Dec;24(12):1945–54.
- 28 Webb BD, Brandt T, Liu L, Jalas C, Liao J, Fedick A, et al. A founder mutation in COL4A3 causes autosomal recessive Alport syndrome in the Ashkenazi Jewish population. *Clin Genet.* 2014 Aug;86(2):155–60.
- 29 Heidt L, Arrondel C, Forestier L, Cohen-Solal L, Mollet G, Gutierrez B, et al. Structure of the human type IV collagen gene COL4A3 and mutations in autosomal Alport syndrome. *J Am Soc Nephrol.* 2001 Jan;12(1):97–106.
- 30 van der Loop FT, Heidt L, Timmer ED, van den Bosch BJ, Leinonen A, Antignac C, et al. Autosomal dominant Alport syndrome caused by a COL4A3 splice site mutation. *Kidney Int.* 2000 Nov;58(5):1870–5.
- 31 Badenas C, Praga M, Tazón B, Heidt L, Arrondel C, Armengol A, et al. Mutations in the COL4A4 and COL4A3 genes cause familial benign hematuria. *J Am Soc Nephrol.* 2002 May;13(5):1248–54.
- 32 Mochizuki T, Lemmink HH, Mariyama M, Antignac C, Gubler MC, Pirson Y, et al. Identification of mutations in the alpha 3(IV) and alpha 4(IV) collagen genes in autosomal recessive Alport syndrome. *Nat Genet.* 1994 Sep;8(1):77–81.
- 33 Bongers EM, de Wijs IJ, Marcelis C, Hoefsloot LH, Knoers NV. Identification of entire LMXB gene deletions in nail patella syndrome: evidence for haploinsufficiency as the main pathogenic mechanism underlying dominant inheritance in man. *Eur J Hum Genet.* 2008 Oct;16(10):1240–4.

- 34 Zhou J, Hertz JM, Tryggvason K. Mutation in the alpha 5(IV) collagen chain in juvenile-onset Alport syndrome without hearing loss or ocular lesions: detection by denaturing gradient gel electrophoresis of a PCR product. *Am J Hum Genet*. 1992 Jun;50(6):1291–300.
- 35 Renieri A, Meroni M, Sessa A, Battini G, Serbelloni P, Torri Tarelli L, et al. Variability of clinical phenotype in a large Alport family with Gly 1143 Ser change of collagen alpha 5(IV)-chain. *Nephron*. 1994;67(4):444–9.
- 36 Wang Y, Sivakumar V, Mohammad M, Colville D, Storey H, Flinter F, et al. Clinical and genetic features in autosomal recessive and X-linked Alport syndrome. *Pediatr Nephrol*. 2014 Mar;29(3):391–6.
- 37 Malone AF, Phelan PJ, Hall G, Cetincelik U, Homstad A, Alonso AS, et al. Rare hereditary COL4A3/COL4A4 variants may be mistaken for familial focal segmental glomerulosclerosis. *Kidney Int*. 2014 Dec;86(6):1253–9.
- 38 Mills KT, Xu Y, Zhang W, Bundy JD, Chen CS, Kelly TN, et al. A systematic analysis of worldwide population-based data on the global burden of chronic kidney disease in 2010. *Kidney Int*. 2015 Nov;88(5):950–7.
- 39 Glasscock RJ, Oreopoulos DG. Aging and chronic kidney disease. *Nephron Clin Pract*. 2011;119(Suppl 1):c1.
- 40 Komatsu H, Kikuchi M, Nakagawa H, Fukuda A, Iwakiri T, Toida T, et al. Long-term survival of patients with IgA nephropathy after dialysis therapy. *Kidney Blood Press Res*. 2013;37(6):649–56.
- 41 Morello R, Zhou G, Dreyer SD, Harvey SJ, Ninomiya Y, Thorner PS, et al. Regulation of glomerular basement membrane collagen expression by LMX1B contributes to renal disease in nail patella syndrome. *Nat Genet*. 2001 Feb;27(2):205–8.
- 42 Hall G, Lane B, Chryst-Ladd M, Wu G, Lin JJ, Qin X, et al. Dysregulation of WTI (-KTS) is Associated with the Kidney-Specific Effects of the LMX1B R246Q Mutation. *Sci Rep*. 2017 Jan;7(1):39933.
- 43 Dreyer SD, Zhou G, Baldini A, Winterpacht A, Zabel B, Cole W, et al. Mutations in LMX1B cause abnormal skeletal patterning and renal dysplasia in nail patella syndrome. *Nat Genet*. 1998 May;19(1):47–50.
- 44 Rafeian-Kopaei M, Nasri H, Alizadeh F, Ataei B, Baradaran A. Immunoglobulin A Nephropathy and Malaria falciparum Infection; a Rare Association. *Iran J Public Health*. 2013 May;42(5):529–33.
- 45 Satoskar AA, Nadasdy G, Plaza JA, Sedmak D, Shidham G, Hebert L, et al. Staphylococcus infection-associated glomerulonephritis mimicking IgA nephropathy. *Clin J Am Soc Nephrol*. 2006 Nov;1(6):1179–86.
- 46 Rollino C, Vischini G, Coppo R. IgA nephropathy and infections. *J Nephrol*. 2016 Aug;29(4):463–8.
- 47 Beaufils H, Jouanneau C, Katlama C, Sazdovitch V, Hauw JJ. HIV-associated IgA nephropathy—a post-mortem study. *Nephrol Dial Transplant*. 1995;10(1):35–8.
- 48 Wu MY, Chen CS, Yiang GT, Cheng PW, Chen YL, Chiu HC, et al. The Emerging Role of Pathogenesis of IgA Nephropathy. *J Clin Med*. 2018 Aug;7(8):7.
- 49 Renieri A, Bruttini M, Galli L, Zanelli P, Neri T, Rossetti S, et al. X-linked Alport syndrome: an SSCP-based mutation survey over all 51 exons of the COL4A5 gene. *Am J Hum Genet*. 1996 Jun;58(6):1192–204.

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