COL4A3/COL4A4 Mutations and Features in Individuals with Autosomal Recessive Alport Syndrome

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ABSTRACT

Alport syndrome is an inherited disease characterized by hematuria, progressive renal failure, hearing loss, and ocular abnormalities. Autosomal recessive Alport syndrome is suspected in consanguineous families and when female patients develop renal failure. Fifteen percent of patients with Alport syndrome have autosomal recessive inheritance caused by two pathogenic mutations in either *COL4A3* or *COL4A4*. Here, we describe the mutations and clinical features in 40 individuals including 9 children and 21 female individuals (53%) with autosomal recessive inheritance indicated by the detection of two mutations. The median age was 31 years (range, 6–54 years). The median age at end stage renal failure was 22.5 years (range, 10–38 years), but renal function was normal in nine adults (29%). Hearing loss and ocular abnormalities were common (23 of 35 patients [66%] and 10 of 18 patients [56%], respectively). Twenty mutation pairs (50%) affected *COL4A3* and 20 pairs affected *COL4A4*. Of the 68 variants identified, 39 were novel, 12 were homozygous changes, and 9 were present in multiple individuals, including c.2906C>G (p.(Ser969*)) in *COL4A4*, which was found in 23% of the patients. Thirty-six variants (53%) resulted directly or indirectly in a stop codon, and all 17 individuals with early onset renal failure had at least one such mutation, whereas these mutations were less common in patients with normal renal function or late-onset renal failure. In conclusion, patient phenotypes may vary depending on the underlying mutations, and genetic testing should be considered for the routine diagnosis of autosomal recessive Alport syndrome.

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Alport syndrome is an inherited renal disease characterized by hematuria, progressive renal failure, hearing loss, and ocular abnormalities. Alport commented in 1927 that the occurrence of hematuria and hearing loss in a pedigree was not coincidental but represented a clinical syndrome, and that the more severe disease in male individuals was consistent with X-linked inheritance.[1] We now understand that nearly 85% of patients have X-linked disease due to a pathogenic mutation in the *COL4A5* gene, and the remaining individuals usually have autosomal recessive inheritance with two pathogenic mutations in either the *COL4A3* or *COL4A4* gene.

Alport syndrome is usually suspected when the typical clinical features are present. Diagnostic features[2] include a positive family history, a lamellated glomerular basement membrane (GBM),[3] high tone sensorineural hearing loss, and lenticonus and macular flecks on ophthalmoscopy.[4] However, these features do not distinguish between X-linked

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and autosomal inheritance. The possibility of autosomal recessive disease is often overlooked, but its recognition is important because the genetic implications are different for the patient and other family members. Affected male individuals with X-linked disease, but few female individuals, eventually develop renal failure and the disease is transmitted from one generation to another. With autosomal recessive inheritance, male and female individuals are equally likely to be affected; renal failure tends to occur in only one generation except in the presence of multiple consanguinity. In our previous report of 206 patients referred for molecular testing of *COL4A5,* the pathogenic mutation detection rates in families fulfilling none, one, two, three, or four diagnostic criteria were 0%, 18%, 64%, 89%, and 81%, respectively. Autosomal recessive inheritance was suspected to account for the families meeting four diagnostic criteria in whom no pathogenic *COL4A5* mutation was detected.[5]

Nearly 300 pathogenic mutations have been described in the *COL4A3* and *COL4A4* genes (Leiden Open Variation Database; https://grenada.lumc.nl/LOVD2/COL4A/home.php?action=switch_db), but many of these are from patients with thin basement membrane nephropathy (TBMN). There are few reports describing two pathogenic mutations in individuals with autosomal recessive Alport syndrome.[6–16] Even fewer studies have examined how mutations may determine clinical features.

Here we describe genetic mutations and clinical features in 40 patients in whom two pathogenic mutations were identified in the *COL4A3* or *COL4A4* gene, consistent with the diagnosis of autosomal recessive Alport syndrome. In many cases, the mutations were demonstrated to be in *trans,* which is on different chromosomes, confirming autosomal recessive inheritance. Testing examined the entire coding region and splice sites of both *COL4A3* and *COL4A4* using unidirectional fluorescent Sanger DNA sequencing, analyzed using Mutation Surveyor software. For detecting point mutations in the regions screened, this approach has an analytical sensitivity and specificity of >99%.[17]

RESULTS

Clinical Features

Between August 2009 and December 2011, 205 apparently unrelated British and Australian patients with clinically suspected autosomal recessive Alport syndrome or TBMN were referred for genetic testing.

Ninety-four individuals (46%) were found to have at least one pathogenic mutation and an additional 36 individuals (17%) had at least one variant of unknown significance. Forty participants (20%) had two pathogenic mutations in *COL4A3* or *COL4A4* (Table 1); in 18 families, these mutations were confirmed to be present in *trans* in the index cases. In 7 of 12 families, dosage analysis for the corresponding exon confirmed the presence of two alleles in individuals apparently homozygous for mutations.

The 40 patients with autosomal recessive Alport syndrome comprised 31 British individuals and 9 Australians, 7 of whom were of British origin. None of the participants was known to be related at the time of testing; however, at a subsequent clinic visit, members of two families recognized each other and the index cases were confirmed to be second cousins (patients 23 and 28).

This cohort included 19 male participants (48%) and 21 female participants (52%). Their median age at the time of study was 31 years (range, 6–54), and nine participants (23%) were aged ≤18 years.

Five of 9 children (56%) and 9 of 31 adults (29%; median age 25.5 years; range, 19–42) had normal renal function, and 20 of 34 patients (59%) had end stage renal failure. The median age at onset of renal failure was 22.5 years (range, 10–38). Within individual families, the age at which affected individuals reached end stage renal failure varied by up to 9 years.

Thirty-four of 36 patients (94%) had a lamellated GBM on renal biopsy. However, two adults who had a biopsy as children (aged 5 and 6 years) had a normal or thinned GBM, respectively. Four patients had no renal biopsy or record of the GBM appearance.

Extrarenal features were common. Twenty-three of 35 individuals (66%) had hearing loss, and 10 of 18 individuals (56%) had lenticonus or retinopathy.

Pathogenic Mutations

Eighty pathogenic mutations were identified (Table 1). We found 68 different variants, with 12 homozygous changes, and 9 variants were present in multiple individuals. Twenty patients (50%) had two pathogenic mutations in the *COL4A3* gene and 20 patients (50%) had mutations in the *COL4A4* gene, while 39 variants were novel (Table 2).

Five individuals were from known consanguineous families (patients 8, 15, 26, 32, and 40) and an additional 7 families were also demonstrated to have homozygous mutations, making a total of 12 families (30%). These included four homozygous *COL4A3* mutations and eight homozygous *COL4A4* mutations. The remaining 28 individuals (70%) had compound heterozygous *COL4A3* or *COL4A4* mutations.

Of the nine variants found on several occasions, five were *COL4A3* mutations and four were *COL4A4* mutations. The *COL4A3* mutations included c.2031_2038dup (p.(Gly680fs)) in two individuals, c.2621_2622delinsT (p.(Gly874fs)) in two individuals,[8] c.2768_2778del (p.(Val923fs)) occurring four times in three individuals, c.3472G>C (p.(Gly1158Arg)) in two individuals, and c.4981C>G (p.(Arg1661Cys)) in three individuals.[8]

The *COL4A4* mutations included c.81_86del (p.(Ile29_Leu30del)) in two individuals, c.1598G>A (p.(Gly533Asp)) four times in two individuals, c.2638del (p.(Ala880fs)) four times in three individuals,[9] and c.2906C>G (p.(Ser969*)) 12 times in nine individuals (23% of our patients). The c.2906C>G (p.(Ser969*)) variant in *COL4A4* was the most common mutation detected in this series, and was described

Table 1. Pathogenic *COL4A3* and *COL4A4* mutations detected in 40 patients with autosomal recessive Alport syndrome

Patient No.	H/h	COL4A3/ COL4A4	Mutation 1	Exon	Novel?	Mutation 2	Exon	Novel?	Sex	Ethnicity	Age (yr)[a]	Age at Renal Failure (yr)	Renal Biopsy	Hearing Loss	Characteristic Eye Signs	Notes
1	h	COL4A3	c.162dup p.(Gly55fs)[b]	3	Yes	c.2313_2330del p.(Leu775_ Gly780del)	30	Yes	M	Tasmanian family, British origin generations ago	34	23	Yes	Yes	Yes	Mutations in *trans*
2	h	COL4A3	c.391G>T	7	Yes	c.2621_ 2622delinsT	32	No, AR AS, H, h[8]	F	Australian family probably originally from UK	38	15	Yes	Yes	Yes	
3	h	COL4A3	p.(Glu131*)[b] c.461G>C	8	Yes	p.(Gly874fs)[b] c.794G>A	14	Yes	M	English	44	37	Yes	No	No	Mutations in *trans*
4	h	COL4A3	p.(Gly154Ala) c.546G>T	9	Yes	p.(Gly265Glu) c.4981C>T	52	No, AR AS, h[8]	F	Australian family, probably originally from UK	38	N/A	Yes	Yes	No	c.546G> T has splice effect
5	h	COL4A3	p.(Gln182His) c.663_664del	12	Yes	p.(Arg1661Cys) c.1937dup	27	Yes	F	English	10	N/A (N @ 9)	Yes	No	NK	Mutations in *trans*
6	h	COL4A3	p.(Arg221fs)[b] c.713del	13	Yes	p.(Glu647fs)[b] c.1918G>A	26	No, AR AS[8]	F	NK	21	N/A (N @ 17)	N @ 6	No	No	
7	h	COL4A3	p.(Pro238fs)[b] c.1085del	19	Yes	p.(Gly640Arg) c.3580del	42	Yes	M	NK	6	N/A (N @ 6)	Yes	No	NK	Mutations in *trans*
8	H	COL4A3	p.(Pro362fs)[b] c.1409–5T>A	22i	No, AR AS[18]	p.(Arg1194fs)[b] c.1409–5T>A	22i	No, AR AS[18]	M	Australian, parents were Italian	54	38	Yes	Yes	Yes	Apparent homozygous. Parents first cousins
9	h	COL4A3	c.2031_2038dup p.(Gly680fs)[b]	28	Yes	c.3472G>C p.(Gly1158Arg)	40	Yes	M	NK	Deceased	26	Yes	Yes	No	
10	h	COL4A3	c.2031_2038dup p.(Gly680fs)[b]	28	Yes	c.3210+1G>A	37i	Yes	M	NK	41	10	Yes	Yes	Yes	
11	h	COL4A3	c.2083G>A	28	No, TBMN[27]	c.2452G>A	31	Yes	M	English	19	N/A (N @ 18)	Yes	No	NK	Mutations in *trans*
12	H	COL4A3	p.(Gly695Arg) c.2567G>A p.(Gly856Glu)	32	Yes	p.(Gly818Arg) c.2567G>A p.(Gly856Glu)	32	Yes	M	NK	16	N/A (N @ 16)	Yes	Yes	No	Apparent homozygous; father heterozygous

Table 1. Continued

Patient No.	H/h	COL4A3/ COL4A4	Mutation 1	Exon	Novel?	Mutation 2	Exon	Novel?	Sex	Ethnicity	Age (yr)[a]	Age at Renal Failure (yr)	Renal Biopsy	Hearing Loss	Characteristic Eye Signs	Notes
13	h	COL4A3	c.2621_2622delinsT p.(Gly874fs)[b]	32	No, AR AS, H and h[8]	c.3866del p.(Gly1289fs)[b]	43	Yes	F	NK	40	17	Yes	Yes	Yes	
14	h	COL4A3	c.2745_2746+7del p.(Gly916fs)[b]	33–33i	Yes	c.4981C>T p.(Arg1661Cys)	52	No, AR AS, h[8]	F	NK	14	NK	Yes	No	No	Mutations in *trans*
15	H	COL4A3	c.2768_2778del	34	Yes	c.2768_2778del	34	Yes	F	Australian family, probably originally from UK	44	25	Yes	Yes	Yes	Confirmed homozygous; consanguineous
16	h	COL4A3	p.(Val923fs)[b] c.2768_2778del	34	Yes	p.(Val923fs)[b] c.4981C>T	52	No, AR AS, h[8]	F	NK	49	NK	NK	NK	NK	Mutations in *trans*
17	h	COL4A3	p.(Val923fs)[b,c] c.2768_2778del	34	Yes	p.(Arg1661Cys) + c.3760G>C	43	Yes	F	Mixed British	36	23	Yes	Yes	Yes	
18	h	COL4A3	p.(Val923fs)[b,c] c.3472G>C	40	Yes	p.(Gly1254Arg) + c.4994G>A	52	Yes	F	English	17	N/A (N @ 17)	TBM@5	No	NK	Mother has one mutation
19	h	COL4A3	p.(Gly1158Arg) c.4030del	46	Yes	p.(Cys1665Tyr) c.4408G>T	48	Yes	M	Punjabi	8	N/A (N @ 7)	Yes	No	NK	Mother has one mutation
20	H	COL4A3	p.(Val1344fs)[b] c.4347_4353del	48	Yes	p.(Gly1470Trp) c.4347_4353del	48	Yes	M	NK	31	17	Yes	Yes	NK	Apparent homozygous. Nonconsanguineous
21	h	COL4A4	p.(Arg1450fs)[b] c.81_86del	3	Yes	p.(Arg1450fs)[b] c.2906C>G	32	No, AR AS, TBMN[9,11,18,19]	F	NK	15	NK	Yes	NK	NK	Father has one mutation
22	h	COL4A4	p.(Ile29_Leu30del) c.81_86del	3	Yes	p.(Ser969*)[b] c.2906C>G	32	No, AR AS, TBMN[9,11,18,19]	M	NK	10	NK	Yes	NK	NK	
23	h	COL4A4	p.(Ile29_Leu30del) c.1099+1G>A	18i	Yes	p.(Ser969*)[b] c.2638del	30	No, AR AS[9]	M	Tasmanian family, likely originally from UK	30	23	Yes	Yes	Yes	
24	H	COL4A4	c.1598G>A p.(Gly533Asp)	22	Yes	p.(Ala880fs)[b] c.1598G>A p.(Gly533Asp)	22	Yes	F	NK	20	N/A (N @ 20)	Yes	No	NK	Confirmed homozygous

Table 1. Continued

Patient No.	H/h	COL4A3/ COL4A4	Mutation 1	Exon	Novel?	Mutation 2	Exon	Novel?	Sex	Ethnicity	Age (yr)[a]	Age at Renal Failure (yr)	Renal Biopsy	Hearing Loss	Characteristic Eye Signs	Notes
25	H	COL4A4	c.1598G>A	22	Yes	c.1598G>A	22	Yes	F	Slovakian	30	N/A (N @ 30)	NK	Yes	NK	Confirmed homozygous Nonconsanguineous
26	H	COL4A4	p.(Gly533Asp) c.1802del	24	No, AR AS[7]	p.(Gly533Asp) c.1802del	24	No, AR AS[7]	F	NK	20	N/A (N @ 20)	Yes	No	No	Apparent homozygous; some consanguinity
27	h	COL4A4	p.(Pro601fs)[b] c.2590G>A	30	No, AR AS[15]	p.(Pro601fs)[b] c.2906C>G	32	No, AR AS, TBMN[9,11,18,19]	F	NK	42	N/A	Yes	No	No	Mutations in *trans*
28	h	COL4A4	p.(Gly864Arg) c.2638del	30	No, AR AS[9]	p.(Ser969*)[b] c.2884G>T	32	Yes	M	Australian	32	28	NK	NK	NK	
29	H	COL4A4	p.(Ala880fs)[b] c.2638del	30	No, AR AS[9]	p.(Glu962*)[b] c.2638del	30	No, AR AS[9]	M	NK	20	NK	Yes	Yes	NK	Confirmed homozygous
30	h	COL4A4	p.(Ala880fs)[b] c.2744del	31	No, AR AS[9]	p.(Ala880fs)[b] c.3197G>T	34	Yes	F	Australian family, likely originally from UK	28	35	NK	NK	NK	
31	h	COL4A4	p.(Gly915fs)[b] c.2906C>G	32	No, AR AS, TBMN[9,11,18,19]	p.(Gly1066Val) c.3215G>T	35	Yes	F	NK	23	17	Yes	Yes	NK	Mutations in *trans*
32	H	COL4A4	p.(Ser969*)[b] c.2906C>G	32	No, AR AS, TBMN[9,11,18,19]	p.(Gly1072Val) c.2906C>G	32	No, AR AS, TBMN[9,11,18,19]	M	Australian, parents were British	34	28	Yes	Yes	Yes	Confirmed homozygous Parents first cousins
33	h	COL4A4	p.(Ser969*)[b] c.2906C>G	32	No, AR AS, TBMN[9,11,18,19]	p.(Ser969*)[b] c.4538G>A	47	Yes	M	NK	39	22	Yes	Yes	NK	
34	h	COL4A4	p.(Ser969*)[b] c.2906C>G	32	No, AR AS, TBMN[9,11,18,19]	p.(Cys1513Tyr) c.4781_4807dup	47	Yes	F	NK	41	N/A (N @41)	Yes	Yes	NK	Mutations in *trans*
35	H	COL4A4	p.(Ser969*)[b] c.2906C>G	32	No, AR AS, TBMN[9,11,18,19]	p.(Ser1594_Leu1602dup) c.2906C>G	32	No, AR AS, TBMN[9,11,18,19]	M	NK	36	18	Yes	Yes	NK	Confirmed homozygous
36	H	COL4A4	p.(Ser969*)[b] c.2906C>G	32	No, AR AS, TBMN[9,11,18,19]	p.(Ser969*)[b] c.2906C>G	32	No, AR AS, TBMN[9,11,18,19]	F	NK	35	NK	Yes	Yes	NK	Apparent homozygous
37	h	COL4A4	p.(Ser969*)[b] c.3602G>A p.(Gly1201Asp)	39	Yes	p.(Ser969*)[b] c.4376G>T p.(Gly1459Val)	46	Yes	F	English	32	N/A (N @ 30)	Yes	No	NK	Sister has one mutation

Table 1. Continued

Patient No.	H/h	COL4A3/ COL4A4	Mutation 1	Exon	Novel?	Mutation 2	Exon	Novel?	Sex	Ethnicity	Age (yr)[a]	Age at Renal Failure (yr)	Renal Biopsy	Hearing Loss	Characteristic Eye Signs	Notes
38	h	COL4A4	c.4200_4201del p.(Gly1401fs)[b]	44	Yes	c.4522G>A p.(Gly1508Ser)	46	Yes	M	Scottish	46	21	Yes	Yes	Yes	Mutations in *trans*
39	h	COL4A4	c.4444dup p.(Leu1482fs)[b]	46	Yes	c.4763G>A p.(Cys1588Tyr)	47	Yes	M	English	29	20	Yes	Yes	NK	Sister has one mutation
40	H	COL4A4	c.4788G>A p.(Trp1596*)[b]	47	Yes	c.4788G>A p.(Trp1596*)[b]	47	Yes	F	NK	17	12	Yes	Yes	NK	Confirmed homozygous–parents are cousins

Sequence nomenclature is based on *COL4A3* reference sequence LRG_230 (NM_000091.4) and *COL4A4* reference sequence LRG_231 (NM_000092.4), in which nucleotide number 1 corresponds to the first base of the translation initiation codon. AR AS, autosomal recessive Alport syndrome; i, intron; F, female; H, homozygous; h, heterozygous; M, male; N, normal; N/A, not appropriate; NK, not known.

[a]Age when clinically assessed.

[b]A pathogenic mutation resulting in a stop codon (*i.e.*, a nonsense or frameshift mutation).

[c]The mutation was originally identified in Germany (Center for Nephrology and Metabolic Disorders, Germany).

previously in both autosomal recessive Alport syndrome and TBMN.[9,11,18,19]

Of the 68 pathogenic mutations detected, 25 (37%) resulted in a missense change, 24 (35%) were frameshift mutations, 12 (18%) were nonsense mutations, 4 (6%) were small deletions/duplications, and 3 (4%) were splicing mutations (Table 1). Of the 24 frameshift mutations, 17 affected *COL4A3* and 7 affected *COL4A4*; of the 12 nonsense mutations, 1 affected *COL4A3* and 11 were found in *COL4A4*, 9 of which were the same c.2906C>G change (Table 2). Of the 25 missense mutations, 15 affected *COL4A3* and 10 were found in *COL4A4*.

The most common missense mutations affected a glycine residue (18 of 25, 72%) and the most common substitution was with arginine (7 of 18, 39%). Interestingly, six of the remaining missense mutations abolished (one mutation in *COL4A3* and two in *COL4A4*) or created (p.(Arg1661Cys) in *COL4A3*) a cysteine residue in the noncollagenous domains.

There were 31 patients (78%) with at least one (n=18, 45%) or two (n =13, 33%) pathogenic mutations that resulted directly, or indirectly through a frameshift, in a stop codon. All 17 individuals who developed end stage renal failure before age 30 years had at least one mutation resulting in a stop codon, with 8 individuals having two mutations. Of the nine adults with normal renal function, three had one mutation resulting in a stop codon (normal renal function at ages 17, 41, and 42 years), and one patient had two (normal renal function at age 20 years). Two of the three patients who developed late-onset renal failure (after 30 years) had no mutations resulting in a stop codon.

The two patients in the study who were second-degree cousins (patients 23 and 28) shared a pathogenic mutation in *COL4A4* [c.2638del; p.(Ala880fs)], but each had a different second pathogenic mutation in *COL4A4* [c.1099+1G>A and c.2884G>T p.(Glu962*), respectively]. The second mutations were not found in any other individuals in this study, and had not been described before. In addition, the families of two unrelated male participants (patients 22 and 38) each comprised three affected brothers, all with autosomal recessive Alport syndrome. Both parents of another patient (patient 17) were each carriers of one of the pathogenic mutations; however, the 74-year-old father had hematuria and anterior lenticonus but normal renal function, possibly due to somatic mosaicism involving the *COL4A3* c.3760G>C (p.(Gly1254Arg)) mutation. (To note, somatic mosaicism is the coexistence of genetically distinct cell populations in a single individual, usually as a result of postzygotic genetic events that can occur at any time in the life cycle. A person who is mosaic for a somatic mutation may or may not be affected by the disorder caused by that mutation. Individuals will express the phenotype depending on how many and which cells are affected. Typically, individuals with somatic mosaicism exhibit a milder phenotype because only a proportion of cells contain the mutation and/or because the mutation is confined to a finite segment of the body.)

Table 2. Summary of pathogenic *COL4A3* and *COL4A4* mutations found in 40 autosomal recessive Alport syndrome patients

Patient/Mutation Features	*COL4A3* Gene	*COL4A4* Gene	Total for All Index Cases
Patients (% of all patients)			
With mutation affecting gene	20 (50)	20 (50)	40 (100)
With homozygous mutations	4 (10)	8 (20)	12 (30)
With compound heterozygous mutations	16 (40)	12 (30)	28 (70)
Total mutations[a]	36	32	68
Previously reported	8	15	23
Novel	28	17	45
Unique mutations	29	20	49
Previously reported	5	5	10
Novel	24	15	39
Mutation type (% of mutations found in that gene)[a]			
Frameshift	17 (47)	7 (22)	24 (35)
Nonsense	1 (3)	11 (35)	12 (18)
Missense	15 (41)	10 (31)	25 (37)
	[10 involving Gly (28)]	[8 involving Gly (25)]	
	[4 involving Cys (11)]	[2 involving Cys (6)]	
Small deletion/duplication	1 (3)	3 (9)	4 (6)
Splicing	2 (6)	1 (3)	3 (4)

[a]Homozygous mutations only counted once.

DISCUSSION

Improved mutation detection techniques for autosomal recessive Alport syndrome have increased the sensitivity of genetic testing. This study demonstrated the pathogenic variants in 40 apparently unrelated individuals with autosomal recessive Alport syndrome. This series represents the largest single cohort of patients with autosomal recessive Alport syndrome in which both pathogenic mutations were identified. It also correlates the genotype with clinical features. Previously, data were published on only 40 patients in whom two pathogenic mutations have been identified.

Clinical data were obtained from the referral forms accompanying samples for testing and were sometimes incomplete. Nevertheless, this study clearly demonstrates the onset of end stage renal failure in autosomal recessive Alport syndrome in both children and in adults even into middle age. Interestingly, renal function remained normal in some adults as well as children. This represents the first description of mild renal disease in autosomal recessive Alport syndrome. Where renal failure occurred, it was more common with a nonsense mutation or a mutation that resulted in a downstream stop codon. Renal failure, hearing loss, and ocular abnormalities were common in both male and female participants. The age at onset of renal failure across the cohort varied more than seen in X-linked Alport syndrome. Most patients had a lamellated GBM on renal biopsy, but one child had thinning only and another had a normal GBM. These changes resemble those seen in X-linked disease, in which minor, sometimes nonspecific, histologic abnormalities are common in early disease.

It was not possible to determine the general mutation detection rate or the numbers of patients referred for specific types of disease, because referral information was often nonspecific; it was unclear how many patients were referred with suspected autosomal recessive Alport syndrome rather than TBMN, autosomal dominant Alport syndrome, or another inherited renal disease. In addition, some patients who may have had autosomal recessive Alport syndrome would not have been included in this analysis because only one pathogenic mutation was detected.

In this study, direct DNA sequencing was used to detect pathogenic mutations, but this technique still overlooks exonic deletions/duplications as well as deep intronic variants. Large deletions and duplications account for approximately 12% of cases of X-linked Alport syndrome, and deep intronic variants in *COL4A5* have also been demonstrated.[20] Although the mutation spectrums of the genes do differ, it seems possible that large deletions/duplications and intronic variants in *COL4A3* and *COL4A4* could account for a number of mutations currently not being detected. However, some patients in which only one mutation was detected may suffer from TBMN, the prognosis of which can be worse than originally thought,[21] rather than Alport syndrome, in which case only one pathogenic mutation would be expected to be detected. The distinction between Alport syndrome and TBMN may be difficult to determine but is critical.[22]

Immunohistochemical analysis of basement membrane type IV collagen expression, with a skin or renal biopsy, has also been reported as a useful diagnostic tool; however, there is a significant false negative rate,[23,24] and renal biopsies are an invasive procedure. In approximately 80% of male individuals with X-linked Alport syndrome, GBM staining for α3(IV), α4(IV), and α5(IV) is completely negative.[25] Although a skin biopsy can be a cost-effective and simple diagnostic test, discordance of expression between the epidermal basement membrane and GBM has been reported.[26] Therefore, immunohistochemistry

is not always reliable and is not widely offered in the United Kingdom.

Most mutations in this cohort were novel and there were no "hot spots" (regions of DNA where mutations are observed with greater frequency) in *COL4A3* or *COL4A4*; however, some mutations recurred in different patients. The high proportion of new mutations suggested that many more are still to be identified. Ten mutations that had been described previously in Alport syndrome or TBMN were present in 22 of the patients (Leiden Open Variation Database, https://grenada.lumc.nl/LOVD2/COL4A/home.php?action=switch_db).[7–9,11,15,18,19,27]

Nine mutations were found more than once. The most common variant, p.(Ser969*), was detected 12 times in nine individuals (23% of patients) and represented 13% (9 of 68) of all mutations in this cohort. This variant was found not only in the British patients but also in the Australians of British origin. This variant has not been reported in other populations, such as the European or Cypriot communities, but appears due to a "founder" effect.[9] Founder mutations have been described in the *COL4A5* gene in North America in X-linked Alport syndrome, and other variants have also been reported in the *COL4A3* and *COL4A4* genes in Cyprus.[28–30]

Only five individuals were known to be from consanguineous families but 12 had homozygous mutations. Two of these mutations, including p.(Ser969*), were found in multiple individuals and the homozygosity likely resulted from the high background prevalence of this variant rather than recent consanguinity.

Nonsense mutations or mutations resulting in downstream stop codons were the most common variants, occurring in 78% of patients (31 of 40). These types of mutations were associated with early onset renal failure. Nonsense mutations result in the loss of collagen IV $\alpha 3\alpha 4\alpha 5$ heterotrimer from the GBM,[31] whereas missense mutations produce a structurally abnormal GBM that is less likely to be deleterious. This study is the first that indicates that the nature of the underlying mutations in autosomal recessive Alport syndrome may also affect the clinical phenotype.

Most missense mutations in this study affected glycine residues and the most common change resulted in an arginine substitution. The p.(Arg1661Cys) mutation in *COL4A3* was previously reported in Alport syndrome[8] and involves an exposed arginine residue that is conserved in all six collagen IV chains. This mutation likely results in mismatched disulphide bond formation, perhaps altering the conformation of the NC1 domain or leading to protomer instability.[8,32] This is likely to be due to a founder effect.[8] Interestingly, several of the missense mutations described here either abolished or created a new cysteine residue. Cysteines in the NC1 domains of collagen IV are highly conserved between and within species and are important for intramolecular disulphide bond formation.[33,34] In addition, the NC1 domain plays an important role in heterodimerization during collagen chain formation and assembly.[35–37]

These genotype-phenotype correlations in autosomal recessive Alport syndrome are similar to those observed in X-linked Alport syndrome, in which mutations that directly or indirectly result in nonsense mutations are associated with early onset renal failure compared with missense mutations that result in less severe disease.[25,38]

When an individual is diagnosed with Alport syndrome, it is important to distinguish between X-linked and autosomal recessive inheritance because of the different risks of renal failure in other family members. Autosomal recessive inheritance is suspected when disease occurs in a consanguineous family, or a single generation, and where male and female individuals are affected with equal frequency and severity. Recessive inheritance may be suspected where a female individual develops renal failure, especially at a young age, because the minority of female individuals with X-linked Alport syndrome who develop renal failure generally do so when they are older (typically in middle age or later). Examination of the pedigree is not always helpful in distinguishing between the likelihood of X-linked and autosomal recessive disease, and may be misleading. This study did not examine the pedigrees of all affected individuals but did identify two families, initially thought to be X-linked, in which all three sons were affected and another family in which the sons of two female cousins were affected and shared only a single pathogenic variant.

Molecular testing confirms the clinically suspected diagnosis of Alport syndrome and is increasingly available. It is appropriate to consider molecular testing instead of, or before, a renal biopsy because the results are conclusive, indicate the mode of inheritance, and avoid the risks associated with an invasive procedure.[39] Once the underlying molecular basis of Alport syndrome is known, the index case and at-risk family members should be offered genetic counseling. Parents of an individual with autosomal recessive Alport syndrome are "obligate carriers" because the new mutation rate is so low. They typically have TBMN and hematuria. Siblings of the index case have a 25% chance of recessive disease too, and carrier couples may wish to consider a range of reproductive options including prenatal and preimplantation genetic diagnosis. Testing can also be used to exclude Alport syndrome in some family members, which allows them to be considered as potential renal donors.

Most apparently sporadic cases of Alport syndrome should first be screened for a pathogenic mutation in *COL4A5* but autosomal recessive inheritance must be considered if no pathogenic variant is identified. Where autosomal recessive inheritance is strongly suspected, it is appropriate to test for pathogenic mutations in *COL4A3* and *COL4A4* in the first instance.

CONCISE METHODS

Study Population

Individuals suspected of having Alport syndrome on clinical features or GBM appearance had been referred by their renal physician or clinical geneticist for genetic testing. Recessive inheritance was suspected in young female participants with renal failure and hearing loss, in the presence of consanguinity, or when disease was observed in

only a single generation. Most participants had previously been tested for mutations in *COL4A5*, with no pathogenic mutation detected.

Genetic testing for pathogenic mutations in *COL4A3* and *COL4A4* was undertaken at a UK accredited reference laboratory. Patients with two pathogenic mutations identified in the *COL4A3* or *COL4A4* genes over a 2-year period (August 2009 to December 2011) were identified and clinical data were collected from the patients' medical records.

Genetic Testing for *COL4A3* and *COL4A4* Pathogenic Mutations

Genomic DNA was extracted from peripheral blood leukocytes using a Chemagic Magnetic Separation Module I and the 3 ml Chemagic DNA Blood Kit Special (PerkinElmer chemagen Technologie GmbH), and the entire coding regions and splice sites of the *COL4A3* (52 exons) and *COL4A4* genes (47 coding exons), sequenced using high-throughput unidirectional tagged primer Sanger sequencing, in a 384-well format, and robotics including the Biomek NX Laboratory Automation Workstation (Beckman Coulter), Multiprobe II 8 Tip Robotic Liquid Handling System (PerkinElmer), and Innovadyne Nanodrop II (IDEX Health and Science). PCR products were generated using Qiagen Multiplex PCR Buffer at an annealing temperature of 56°C, and cleaned using AMPure magnetic beads (Agencourt Bioscience Corporation). Sequencing used a BigDye Terminator v3.1 cycle kit (Applied Biosystems) and products were cleaned with CleanSEQ magnetic beads (Agencourt Bioscience Corporation). The sequence was read with an ABI Prism 3730 DNA analyzer (Applied Biosystems) and analyzed using Mutation Surveyor software (SoftGenetics). Any nonpolymorphic variants were confirmed using a fresh DNA dilution by bidirectional tagged primer Sanger DNA sequencing. Screening of DNA for whole gene or exon deletions and duplications was not carried out. Where possible, relatives of the index cases were examined for a heterozygous copy of the mutation to confirm that both mutations were present in *trans*.

Dosage Analyses

PCR products were generated with unlabeled, tagged gene-specific primers and fluorescently labeled tag primers in Qiagen Multiplex PCR Buffer at an annealing temperature of 56°C. Products were detected using an ABI Prism 3730 DNA analyzer (Applied Biosystems), and the fragments were examined with GeneMarker software (SoftGenetics). The dosage quotient analysis calculation was performed with Microsoft Excel spreadsheet software.

Variant Nomenclature and Classification

Variants were described according to the *COL4A3* reference sequence LRG_230 (NM_000091.4) and *COL4A4* reference sequence LRG_231 (NM_000092.4), where nucleotide number 1 corresponds to the first base of the translation initiation codon, and using the nomenclature recommended by the Human Genome Variation Society.[40]

Variant pathogenicity was assessed using Alamut software (versions 1.5 and 2.0; Interactive Biosoftware), which includes tools AlignGVGD, SIFT, and Polyphen, interrogation of dbSNP and the Swissprot databases, interspecies conservation, variant domain location and Grantham distance due to the effect of the mutation, a Google web search, and splicing algorithms SpliceSiteFinder-like, MaxEntScan, NNSplice, and GeneSplicer. Pathogenicity of variants was determined in accordance with the Clinical Molecular Genetics Society best practice guidelines.[41]

Laboratory and online disease databases were also examined to determine whether the variant had been reported previously and family members were tested where possible to determine whether the variant segregated with disease within the family. All variants were recorded in the *COL4A3* and *COL4A4* Leiden Open Variation Databases (http://www.lovd.nl/COL4A3 and http://www.lovd.nl/COL4A4).[42,43]

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DISCLOSURES

None.

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See related editorial, "The Interface of Genetics with Pathology in Alport Nephritis," on pages 1925–1927.