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X-Linked Alport Syndrome: an SSCP-Based Mutation Survey over All 51 Exons of the COL4A5 Gene

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Summary

The COL4A5 gene encodes the α5 (type IV) collagen chain and is defective in X-linked Alport syndrome (AS). Here, we report the first systematic analysis of all 51 exons of COL4A5 gene in a series of 201 Italian AS patients. We have previously reported nine major rearrangements, as well as 18 small mutations identified in the same patient series by SSCP analysis of several exons. After systematic analysis of all 51 exons of COL4A5, we have now identified 30 different mutations: 10 glycine substitutions in the triple helical domain of the protein, 9 frameshift mutations, 4 in-frame deletions, 1 start codon, 1 nonsense, and 5 splice-site mutations. These mutations were either unique or found in two unrelated families, thus excluding the presence of a common mutation in the coding part of the gene. Overall, mutations were detected in only 45% of individuals with a certain or likely diagnosis of X-linked AS. This finding suggests that mutations in noncoding segments of COL4A5 account for a high number of X-linked AS cases. An alternative hypothesis is the presence of locus heterogeneity, even within the X-linked form of the disease. A genotype/phenotype comparison enabled us to better substantiate a significant correlation between the degree of predicted disruption of the α5 chain and the severity of phenotype in affected male individuals. Our study has significant implications in the diagnosis and follow-up of AS patients.

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Introduction

Alport syndrome (AS) is characterized by a progressive glomerulonephritis with typical ultrastructural changes in the glomerular basement membrane, often associated with high-tone hypoacusis and minor ocular lesions (Flinter et al. 1988). AS is genetically heterogeneous, caused by mutations in at least three genes encoding different chains of type IV collagen: α3, α4, and α5 (Barker et al. 1990; Lemmink et al. 1994; Mochizuki et al. 1994). Each of these genes is composed of >50 exons, rendering mutation detection a very difficult task.

The most frequent type, X-linked AS, is due to mutations in the COL4A5 gene (Barker et al. 1990). A wide allelic heterogeneity exists in X-linked AS, because the large majority of patients bear a different COL4A5 mutation. A total of 5%–10% of the cases are large gene rearrangements involving different regions of the COL4A5 gene (Tryggvason et al. 1993). In rare cases, these deletions involve both the COL4A5 and the flanking COL4A6 gene, resulting in a phenotype of AS associated with diffuse leiomyomatosis (Zhou et al. 1993; Renieri et al. 1994*a*; Heidet et al. 1995).

Mutation analysis of the COL4A5 gene has been performed by several groups, including ours, and ~50 different mutations have been identified (Tryggvason, in press). However, a systematic analysis of the entire coding region of the gene was not performed in any of these studies. We have elsewhere reported the identification of 9 large rearrangements and 18 point mutations in the COL4A5 gene in 27 unrelated Italian individuals affected with AS (Renieri et al. 1992, 1993, 1994*b*, 1994*c*, 1995; Massella et al. 1994; Peissel et al. 1994; Turco et al. 1995). Here, we report our extended analysis on 201 unrelated patients on all 51 exons of the COL4A5 gene. In this study, we also included individuals in whom the diagnosis of AS was not certain, to verify the power of molecular testing with respect to clinical diagnosis. Our results help in the design of a

better strategy for mutation identification in X-linked AS and facilitate genotype-phenotype correlations.

Patients, Material, and Methods

Patients

DNAs of 201 AS families were collected from 51 Italian nephrology and medical genetics centers. A diagnostic score (certain, likely, possible, or doubtful) was assigned to each proband on the basis of the clinical and genetic data at submission: typical glomerular basement membrane thickening and splitting in a hematuric patient was considered certain AS; familial progressive renal damage with high-tone hearing loss, likely AS; isolated familial progressive renal damage or the association of hematuria with high-tone hearing loss, possible AS. The association of sporadic hematuria with hearing loss, not limited to high tones, or with doubtful renal histology, was classified as doubtful AS. Following these criteria, diagnosis was certain in 64 (32%) of the probands, likely in 66 (33%), possible in 42 (21%), and doubtful in 29 (14%).

In 129 families (64.5%), inheritance was consistent with an X-linked semidominant pattern; 1/3 of these had >5 affected members (male or female), 1/3 had 3 or 4, and 1/3 had only 2 (generally mother and son). Thirty-seven cases (18%) were sporadic. In 19 families (9%), 9 of which had certain or likely AS, the most likely inheritance pattern was autosomal recessive, and, among 6 with a seemingly autosomal dominant pattern, diagnosis was certain or likely in 2. The pattern of inheritance was not determinable in 10 cases. Overall, the exact age at end-stage renal disease (ESRD) in males was known in 76/201 families. In 28 (37%) of these, it was $\geq$31 years (average 40 $\pm$ 7.3 years) and in 48 (63%), it was <31 years (average 20 $\pm$ 5.5 years).

SSCP Screening

The 51 exons were divided into 49 PCR products, exons 11–12 and 14–15 being amplified together (table 1). Primers were in most cases designed on intronic sequences: for exons 33–51 from Zhou et al. (1991), for exons 1, 5, 6, 8–10, and 31 from Zhou et al. (1994), for exons 3, 4, 7, 32, and 11–30 from unpublished sequences. Exons 2 and 37 were amplified with primers tailored on the cDNA, because intronic sequences are not available; therefore, mutation screening skipped the ends of these two exons. In the general PCR protocol for exon amplification, 100 ng genomic DNA, mixed with 12.5 pmol primers, 5 nmol dNTP 0.1 U *Taq* polymerase, 3 mM of $MgCl_2$ in 25 μl total volume underwent 5 min denaturation at 94°C, followed by 35 cycles with 1 min at 94°C, 1 min at the annealing temperature shown in table 1, 1 min at 72°C, and 7 min final extension at 72°C. Protocol variants for some exons are detailed in table 1. PCR product length varied from 60 bp to 300 bp. SSCP analysis was performed according to the original isotopic method of Orita et al. (1989) for exons 4, 13, 14–15, 17, 19, 20–25, 30, 46, 47, and 49, or otherwise performed with modified nonradioactive techniques: ethidium-bromide staining (Yap and McGee 1992) for exons 3, 9, 28, 34, 35, 43, 44 (4 μl of the PCR product run on 10% acrylamide gel at 4°C without glycerol and at 20°C with glycerol), and silver staining (Dockhorn-Dworniczak et al. 1991) for the remaining 29 exons (6% acrylamide gel with 10% glycerol run at 4°C). All shifts identified in a given PCR product were first reconfirmed by an independent PCR, and shifts that could not be reconfirmed were considered as PCR-induced mutations; such errors occurred in 10 cases during the entire screening. Sequencing of the variant band then followed, in most cases directly on the purified PCR product (~0.2 pmol) by use of a commercial kit (Sequenase version 2.0, USB), with both the sense and the antisense PCR primer. Some ambiguous sequences (generally from females) required cloning in pUC19 vector. Automated sequencing was performed on an a.L.F. DNA sequencer (Pharmacia), using the autoread sequencing kit (Pharmacia).

Results

All patients were previously screened for major COL4A5 and COL4A6 gene rearrangements (Renieri et al. 1995). Moreover, preliminary studies on some of the exons led to identification of 18 small mutations. SSCP screening of all 51 exons allowed us to identify a causative mutation in 33 additional patients (table 2). All mutations were unique, except for G866E, G869R, and 1685del36, which were found in two unrelated families, and are scattered throughout the entire gene (fig. 1). In most families, segregation of mutation with the disease was tested by either SSCP or restriction-site change (table 2) in at least two affected relatives. Five de novo mutations were identified, in addition to the two we have previously reported (Massella et al. 1994): four (YYY, LSC, FER, and CNT) arose in one of the parents' gametes, while the fifth (DGU), which was inherited from the mother, could be traced to have originated in the grandfather's gamete.

Missense Mutations

Substitutions of glycines in the collagenous region are supposed to be pathogenic because they create a kink in the folding of the triple helix. Ten glycine codon changes were detected in exons 12, 19, 20, 22, 24, 31, and 45, in addition to the ones we have previously reported in five exons (Renieri et al. 1992, 1994*c*; Turco et al. 1995; and T. Neri and P. Zaanelli, unpublished data) (fig. 2). Some of these mutations affect consecutive glycine residues (see table 2). In two cases, the same mutation was detected in two apparently unrelated families: G866E in

Table 1

Primer Sequencee and PCR Conditions

Exon	Primer Sequence (5′→3′)	Product Length (bp)	Annealing Temperature (°C)	Variant Conditions
1	TGTCCCTCTCCGGCTCTAGC	215	62	...
	GGAGGGGAGGAAGGACTTAC			
2	GCTTGCTATGGGTGTTC	60	50	30-s extension
	CTTTTCCCCTTTTATGC			
3	TCTCAACCATGCCTGTGCTT	250	62	30-s extension
	TGATGTGACACCTACTCCCAC			
4	TCACAGATGTTTACAGTAGTTTAAA	250	56	30-s extension
	GGTCTTTTCCAATTGTCTGAT			
5	GATTTTATTTCTTCTTATAG	85	42	...
	AAAAGTGAAATGCTACTTA			
6	TGTTATGTCGCTTTTCAA	103	42	...
	ATTAAATGGATGGATCTC			
7	GGAAAGTGAAGGCTAATGAAC	300	60	...
	GCATTGGGCTCTCTCACTACA			
8	CCTTTTCTTTTTAATAATAG	67	40	...
	CCCCAGAATGAGATG			
9	CCATTGATGGCTTCTTTTA	121	46	30-s extension
	GAAAATCACTGGATACTTA			1.5 mM $MgCl_2$
10	CTTTACTCACTTTATAACAGG	99	52	1.25 mM $MgCl_2$
	AAACAACACAGCTTACCATC			
11–12	TCTTTTGTCTTCTCTTCTTAG	213	52	...
	AACCAGCTCTCTTTCTTTAC			
13	CACTGTCTTATTTTATCTTGC	144	52	...
	TTGACTTCCTCCCTACTTAC			
14–15	TTCCTTTCCCCTACTACTGC	259	58	...
	CAAAGAATATTAGCAGTTACATCAC			
16	TTGCCCTATCATTTCTTTGTATC	108	52	...
	CAGGGGGAAAGAACCTTAGC			
17	ATGTCACCCTATCCTCTATG	109	52	...
	GTCACTTCAAAGTATTAAAATTC			
18	CTATTTTACAATTGCATTG	101	52	...
	ATTTGATAAACGAAGACTA			
19	ATTTTTTTTTTCTTTGGTAATAAAG	184	54	...
	AAGGCCATAAATGCAATCTC			
20	GATGAAATCATTTTGATCAC	235	52	...
	CTTAATAGGAGAAAAATATAGC			
21	GCTATCCTTTCTTTATCTTAC	133	52	...
	AATGAATATGTTTGGAGATC			
22	TGTTATTATGATTTCACTAG	142	50	...
	TTAGAAGGTTACCCTGAGGC			
23	GTTAGGATCTCTTGGTTTCC	110	54	...
	CTTCTTTTTGTTTTATAATATACTC			
24	TTTTTTTCCTTACTCATTTC	236	54	...
	CAAAAATATCAAACCAACTC			
25	ATATGTTTCTGTATTAAAC	226	50	...
	TAAAACAAAACACAGTAAAC			
26	TTTACCATTGATTTACTCTTGC	150	54	...
	AGTTACTTTGAAATAAATTCCTC			
27	CTTTCAATAACTGCTGTTTCTCC	156	52	...
	TGCCTGCTACCCATTCCAAC			
28	TTTGGTGGTTAAAAAATGAC	161	48	1.5 mM $MgCl_2$
	GAGAAGGAATAAAGAAAAATG			
29	GTGTTTTGTCATGTGTATGCTC	201	60	...
	TGAGATGCAGTGACAGCCTC			
30	TTTTCTTGCTGAATGAATGC	300	55	...
	CACTTTATTGATGAGCTAAC			

(*continued*)

Table 1 (continued)

Exon	Primer Sequence (5′→3′)	Product Length (bp)	Annealing Temperature (°C)	Variant Conditions
31	ATTGATATTGTATTAACT GAAAACTTTAAACAAATT	208	42	...
32	AATAGTTTTCTGGTTGACATC TATTCTGTACTGACATAAAGC	250	55	...
33	AATATTCATAAATAAATTCATTCACT ATATTGTGTTTTCACACACTTTG	210	52	...
34	ACTTGCCTCTTCTACTCA ATGGCCTATCACACTTAC	146	50	...
35	ACCAATTTGACCTTTCTA TATTTGGAAGATTTTCAT	134	47	...
36	AATATTATATATCACATATTTTC ATGTGCCTAAAACTATATGCC	198	51	...
37	GGTGAGCCTGGTCTGCCTG CTGGGAATCCAGGAAGGCC	127	62	10% dimethysulfoxide, hot start
38	ATTTTTAAATTGAGCTCTTTACTC ACAGCAAACTGTTATTTTTCAT	136	52	...
39	TAACCTGCTGTACTCAATTTT AAATAGGAAAAATGAAAAACTACA	152	52	...
40	TGATTTAGCATGTTTTATTAAGG GTTTTGTTTTGTTTTGTACTCTG	105	52	...
41	TTATCTTCTAATTATACTTTACTTTC AGACCATTCTCCTACCACTC	252	56	...
42	ATGTCGTCATTTGCTGTG TCTACTTCCATTTCCTAC	181	49	...
43	TTTGTAACATTAATGATTTTATT GAAACCACAAAAATCCCTTC	129	55	...
44	TGACTGGGTAAAGGTTGTAG TAACTATCTTCAGGAATAAGTC	160	55	...
45	AATTTGTGTGTTTTGTCTCA TAATAAAGATGATCTGCATTG	177	52	...
46	ATTTGAATGCCTCATTCTTTTCCT CACCAACAGCATGTTTTACTTGTC	155	58	...
47	TCTTGTATACTGATTATTTCGTGG AGTAGGAAATTAGATATTGATTAT	272	58	...
48	CTGTTTTCTCTCCAAATCTT AAGTCACAGCTAAATCAATG	229	52	...
49	ATTATGTTCCTTCTCCTTTTCCTT ATGACAAATGCAAGGAAGAGTGT	175	58	...
50	GCGGCACATTTTTCCTTGTC GGACCTGAATTAAAGCTATAAGCAC	231	52	...
51	GATCTGATTGTCTTATTTCTTAT ACAAAAGGAATTCTTCAAAATG	134	52	...

NOTE.—Exonic sequences are underlined.

FPR and SLU and G869R in GTT and LUC. The latter mutation was previously described in an English family (Boye et al. 1995). Haplotype analysis using intragenic polymorphic markers suggests that this mutation originated independently in these two Italian families (data not shown).

Insertion/Deletion Mutations with Frameshift

Nine frameshift mutations were found in exons 11, 20, 23, 26, 37, 43, and 47, in addition to the 6 that were reported elsewhere (Renieri et al. 1993, 1994*b*; Massella et al. 1994; Peissel et al. 1994). These mutations are expected to reduce the mRNA level or to result in truncated proteins of different lengths (fig. 3), if enough mRNA is retained.

In-Frame Deletions

Four in-frame deletions were found in exons 21, 22, 33, and 44 spanning the triple helix domain (table 2). Two of these deletions (ΔS942 and ΔPG1351) disrupt

Table 2

Mutations Identified in the COL4A5 Gene

Name	Nucleotide Change	Effect on Coding Sequence	Predicted Effect on Protein	Exon	Restriction Enzyme	Family	Sex	Age (years)	Age at ESRD (years)
Initiator codon:									
1 M1V	A→G at 203	Met→Val at 1	Start change	1	...	AGO	M	48	28
Missense:									
2 G54D	G→A at 363	Gly→Asp at 54	Interrupts continuous Gly-X-Y	3	−*Bst*NI	BAL	M	47	40
3 G174R	G→C at 722	Gly→Arg at 174	Interrupts continuous Gly-X-Y	9	...	ESP	...	...	...
4 G177R	G→C at 731	Gly→Arg at 177	Interrupts continuous Gly-X-Y	9	−*Ava*II	FPZ	M	45	34
5 G219S	G→A at 857	Gly→Ser at 219	Interrupts continuous Gly-X-Y	12	...	XXX	M	18	–
6 G325R	G→A at 1175	Gly→Arg at 325	Interrupts continuous Gly-X-Y	17	−*Msp*I	BAD	M	36	>36 (CRF)
7 G325E	De novo G→A at 1176	Gly→Glu at 325	Interrupts continuous Gly-X-Y	17	−*Msp*I	VIZ	M	9	–
8 G371E	G→A at 1314	Gly→Glu at 371	Interrupts continuous Gly-X-Y	19	...	PRI	M	31	19
9 G374A	G→C at 1323	Gly→Ala at 374	Interrupts continuous Gly-X-Y	19	...	PTU	M	9	–
10 G406V	G→T at 1419	Gly→Val at 406	Interrupts continuous Gly-X-Y	20	...	ARG	M	...	31,5[a]
11 G409D	G→A at 1428	Gly→Asp at 409	Interrupts continuous Gly-X-Y	20	...	ROS	M	16	>16 (CRF)
12 G494D	G→A at 1683	Gly→Asp at 494	Interrupts continuous Gly-X-Y	22	−*Hin*dII	CAC	M	20	–
13 G567A	G→C at 1902	Gly→Ala at 567	Interrupts continuous Gly-X-Y	24	−*Sac*I	GAL	M	39	16
14 G866E	G→A at 2799	Gly→Glu at 866	Interrupts continuous Gly-X-Y	31	−*Bam*HI	FPR	M	31	>31 (CRF)
						SLU	M	7	–
15 G869R	G→A at 2807	Gly→Arg at 869	Interrupts continuous Gly-X-Y	31	−*Msp*I	GTT	M	12	10
						LUC	M	13	–
16 G1143S	G→A at 3629	Gly→Ser at 1143	Interrupts continuous Gly-X-Y	38	−*Msp*I	COL	M	...[a]	39[a]
17 G1379V	G→T at 4338	Gly→Val at 1379	Interrupts continuous Gly-X-Y	45	−*Hin*fI	DBT	...	...	...
18 R1410C	C→T at 4430	Arg→Cys at 1410	Additional Cys next to NC	46	...	SPN	M	...	26,5[a]
19 G1421W	G→T at 4463	Gly→Trp at 1421	Interrupts continuous Gly-X-Y	46	...	BIN	M	33	>33 (CRF)
20 P1517T	C→A at 4751	Pro→Thr at 1517	Changes conserved aa in NC	48	...	PST	M	15	–
21 G1596D	G→A at 4989	Gly→Asp at 1596	Changes conserved aa in NC	49	...	DVA	M	19	–
Frameshift:									
22 836delC	Deletion of C at 836-837	Frameshift from 211	Termination 8 codons on	11	−*Mva*I	ZPA	M	28	21
23 1419insGGGG	Insertion of GGGG after 1419	Frameshift from 406	Termination 5 codons on	20	...	DIM	M	19	>19 (CRF)
24 1482insA	Insertion of A at 1482	Frameshift from 427	Termination 20 codons on	20	...	NER	M	26	23
25 1768delA	Deletion of A at 1768-1769	Frameshift from 523	Termination 33 codons on	23	...	ZFE	...	...	...
26 2220delG	De novo deletion of G at 2220	Frameshift from 673	Termination 4 codons on	26	...	YYY	...	...	...
27 3142delA	Deletion of A at 3142-3145	Frameshift from 980	Termination 15 codons on	34	...	ORL	M	...[a]	16[a]
28 3532delA	Deletion of A at 3532-3533	Frameshift from 1110	Termination 41 codons on	37	+*Ava*II	DNI	...	...	...
29 3539insCCTG	De novo insertion of CCTG after 3539	Frameshift from 1112	Termination 24 codons on	37	...	LSC	M	3	–
30 3710delG	Deletion of G at 3710-3711	Frameshift from 1170	Termination 128 codons on	39	−*Msp*I	SCA	M	19	>19 (CRF)
31 3745del7	Deletion of 7 bp from 3745	Frameshift from 1181	Termination 115 codons on	39	...	RMA	M	26	19
32 3820delTGGA	Deletion of TGGA from 3820	Frameshift from 1206	Termination 91 codons on	41	...	DGR	M	...[a]	22[a]

Hypoacusis	Ocular Changes	Sex	Age (years)	Age at ESRD (years)	Hypoacusis	Ocular Changes	Reference	Reference, Other Families with Same Mutation
?	Maculopathy	F	23	–	–	–	Present study	–
+	Lens opacity	F	16	–	–	–	(Turco et al. 1995)	–
...	...	F	31	–	+	–	(Neri et al. unpublished)	–
+	Myopia	F	55	>55 (CRF)	+	–	(Neri et al. unpublished)	–
–	–	F	46	–	+	–	Present study	–
+	?	F	8	–	–	–	(T. Neri and P. Zanelli, unpublished data)	(Knebelmann et al. 1992)
–	–	...	...	...	...	...	(Renieri et al. 1992)	–
+	Maculopathy	F	35	–	+	–	Present study	–
–	?	...	...	...	...	...	Present study	–
+	Cataracts	...	...	...	...	...	Present study	(Boye et al. 1995)
+	...	F	51	>51 (CRF)	?	?	Present study	–
+	Maculopathy	...	...	...	...	...	Present study	–
+	–	F	59	–	+	–	Present study	–
+	?	...	...	...	...	...	Present study	–
–	–	...	...	...	...	...	Present study	–
–	–	F	33	–	–	–	Present study	(Boye et al. 1995)
+	Lens opacity	F	?	30	?	?	Present study	–
+	Lens opacity	F	66	63	+	Lens opacity	(Renieri et al. 1994*c*)	–
...	...	F	12	–	–	–	Present study	–
+	–	...	...	...	...	...	(Neri et al. unpublished)	–
–	–	...	...	...	...	...	(Neri et al. unpublished)	–
–	–	...	...	...	...	...	(Neri et al. unpublished)	(Lemmink et al. 1993)
+	–	F	Dead	23	?	?	(Neri et al. unpublished)	–
+	–	F	57	–	–	?	Present study	–
+	Anterior lenticonus	F	38	–	–	?	Present study	–
+	Maculopathy	F	52	30	+	Maculopathy	Present study	–
...	...	F	50	31	+	?	Present study	–
...	...	F	16	–	–	–	Present study	–
+	Lenticonus	F	31	28	+	–	(Peissel et al. 1994)	–
...	...	F	61	47	?	?	Present study	–
–	–	...	...	...	...	...	Present study	–
+	–	F	51	–	–	–	(Renieri et al. 1994*b*)	–
+	–	F	50	–	–	–	(Renieri et al. 1993)	–
+	Anterior lenticonus	F	63	>63 (CRF)	+	?	(Renieri et al. 1993)	–

(*continued*)

Table 2 (continued)

Name	Nucleotide Change	Effect on Coding Sequence	Predicted Effect on Protein	Exon	Restriction Enzyme	Family	Sex	Age (years)	Age at ESRD (years)
33 4016delC	Deletion of C at 4016-4017	Frameshift from 1272	Termination 26 codons on	42	+*Dde*I	TOB	M	28	20
34 4193delT	Deletion of T at 4193-4194	Frameshift from 1331	Termination 3 codons on	43	+*Bam*HI	GLN	M	8	–
35 4363insTCCT	De novo insertion of TCCT after 4363	Frameshift from 1388	Termination 30 codons on	45	...	FUL	...	...	...
36 4546insT	De novo insertion of T after 4546	Frameshift from 1449	Termination 36 codons on	47	...	DGU	M	14	12
37 5033delG	Deletion of G at 5033-5034	Frameshift from 1611	Termination 3 codons on	50	...	MIB	M	17	12
Nonsense:									
38 R373X	C→T at 1319	Arg→Stop at 373	Termination at 373	19	...	MAC	M	16	15
In-frame deletion/ insertion:									
39 ΔPGP456	De novo deletion of 9 bp from 1567	Deletion of Pro-Gly-Pro	Chain shortening	21	...	CNT	...	...	...
40 1685del36	Deletion of 36 bp from 1685	Deletion of 12 amino acid	Chain shortening	22	...	FAN	M	...[a]	22[a]
						CIV	M	31	27
41 ΔS942	De novo deletion of TAG between 3025 and 3028	Deletion of Ser942	Interrupts continuous Gly-X-Y	33	...	FER	...	...	...
42 ΔPG1350-1351/G1348E	Deletion of 6 bp from 4245 and G→A at 4241	Deletion of Pro-Gly 1350-1 and Gly→Glu at 1348	Interrupts continuous Gly-X-Y	44	...	LZP	M	25	–
43 4517dupl36	Duplication of 36 bp from 4517	Duplication 12 amino acid from Gly 1439	Chain elongation	47	...	RUS	...	...	...
Splicing:									
44 1367+1G→A	G→A at 1367+1	5′ splice signal	Includes intron 19	Intron 19	...	CRT	M	19	–
45 1367+2T→G	T→G at 1367+2	5′ splice signal	Includes intron 19	Intron 19	...	CRD	M	27	13
46 1981+1G→T	G→T at 1981	5′ splice signal	Includes intron 24	Intron 24	...	SES	...	...	...
47 2597+2delT	Deletion of T at 2597+2	5′ splice signal	Includes intron 29	Intron 29	...	CGA	M	27	17
48 4200-2A→G	A→G at 4200-2	3′ splice signal	Skips exon 44	Intron 43	−*Bsg*I	TRU	M	52	30

NOTE.—Nucleotide and amino acid numbering according to (Zhou et al. 1994); a plus sign (+) indicates presence of a characteristic; a minus sign (−) indicates the absence of a characteristic; ellipses (. . .) indicate absence of data.

[a] Mean age among several related males.

the leitmotif Gly-X-Y. A 36-bp deletion involves a 6-bp direct repeat, one copy of which spans the intron 22 5′ splice site. We cannot establish whether this deletion has a deleterious effect on splicing, since a canonical consensus sequence is present in the retained copy of the repeat. This mutation was detected in two apparently unrelated families (FAN and CIV). Haplotype analysis using intragenic polymorphic markers suggests that this mutation may have originated from a common ancestor (data not shown).

Splice-Site Mutations

Five splice-site mutations were found each in introns 19, 24, 29, and 43. Four affected the 5′-GT, while one affected the 3′-AG splice signals (table 2).

Nonsense and Start-Codon Mutations

A single nonsense mutation was detected in exon 19, changing codon 373 from an arginine to a stop; an identical mutation was previously reported (Heiskari et al., in press). An A-to-G transition within the ATG start codon was the only mutation detected in the coding portion of exon 1. No alternative ATG codons are present in the first six exons; therefore, this mutation is believed to alter translation of the protein.

Rare Variants

Three apparently nonpathogenic nucleotide substitutions were found to cosegregate with AS, in addition to those reported elsewhere (Renieri et al. 1995). Two of these new mutations affect the third position of the codon (2497A→T and 2562 A→G), and one lies within an intron (640+47A→G). Synonymous changes are not supposed to be pathogenically involved and are accordingly named "silent." However, some caution must be kept in considering these synonymous mutations as neutral, because they might hide cryptic splice sites (Richard and Beckmann 1995) and/or affect mRNA stability.

Hypoacusis	Ocular Changes	Sex	Age (years)	Age at ESRD (years)	Hypoacusis	Ocular Changes	Reference	Reference, Other Families with Same Mutation
+	?	F	?	50	+	?	(Renieri et al. 1994*b*)	–
Trasm	–	F	28	26	–	?	Present study	–
...	...	F	16	–	–	–	(Massella et al. 1994)	–
+	–	F	42	39	+	–	Present study	–
+	–	F	50	–	+	?	(Renieri et al. 1993)	–
+	–	F	36	–	–	Myopia	Present study	(Heiskari et al., in press)
...	...	F	14	–	–	–	Present study	–
+	–	...	...	...	...	...	Present study	–
+	–		51	47	+	–	Present study	–
...	...	F	22	–	–	Anterior lenticonus	Present study	–
+	–	F	53	–	+	–	Present study	–
...	...	F	14	–	–	Lens opacity	(Hamalainen et al., in press)	–
+	Corneal dystrophy	F	43	41	–	–	Present study	–
+	Myopia	F	53	–	–	–	Present study	–
...	...	F	30	–	–	?	Present study	–
+	Maculopathy	F	49	–	?	–	Present study	–
?	?	F	75	>75 (CRF)	–	?	Present study	–

Discussion

The entire COL4A5 coding sequence was analyzed by SSCP analysis on genomic DNA from a clinically heterogeneous population of 201 AS patients. We detected a mutation in 60 patients, including cases described in our previous reports (Renieri et al. 1992, 1993, 1994*b*, 1994*c*, 1995; Massella et al. 1994; Peissel et al. 1994; Turco et al. 1995). Among these 201 patients, only 96 had a certain/likely diagnostic score and an X-linked semidominant inheritance. A causative mutation was found in 43 (45%) of the 96 families (table 3). The remaining 53 families showed X-linked semidominant pattern of inheritance and 1/3 of these had more than five affected members (male or female), 1/3 had three or four, and 1/3 had only two (generally mother and son) as mutated families did. Thus, in ~50% of patients with a "certain" and "likely" diagnosis of X-linked AS, the molecular defect remains unknown. Our failure to detect mutations in these individuals can only be ascribed in part to technical limitations of SSCP analysis, which is thought to have a mutation-detection rate of ~80% (Grompe 1993). Our analysis focused exclusively on the coding region, and therefore we could not detect mutations in the promoter or introns or in the additional alternatively spliced exon (Guo et al. 1993). A similar situation was observed in other diseases, such as hemophilia A and ocular albinism type 1 (Schiaffino et al. 1995). In hemophilia A, this puzzle was recently solved by the finding of a common mutation: a previously undetected Xq terminal inversion, which leads to a disruption of factor VIII mRNa in ~50% of the patients with a severe disease (Lakich et al. 1993) . An alternative explanation of the low rate of detected mutations could be the involvement of other gene(s), even within the X-linked form. It is possible that muta-

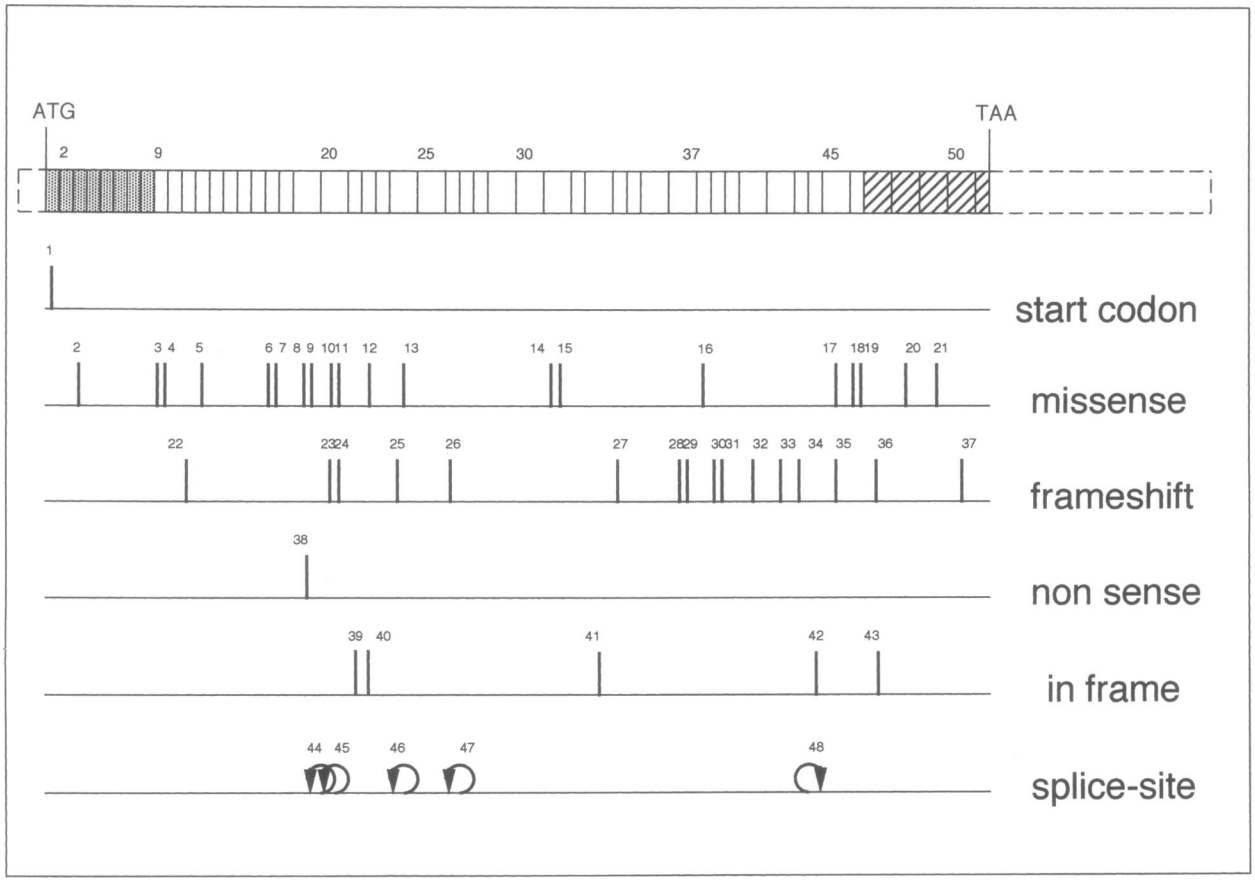

Figure 1 Distribution of mutations in COL4A5 gene. The coding region is divided in the 51 exons (boxes). UTRs (hatched boxes) were not analyzed. Exons encoding the 7S domain (gray boxes) and the NC domain (striped boxes) are shown. Each vertical bar represents the location of a mutation, which is labeled as in table 2. For frameshift mutations, the last unaltered codon is indicated.

tions in the COL4A6 gene, adjacent to COL4A5, account for some cases of AS. However, a search of COL4A6 mutations in AS patients has so far been unsuccessful (Renieri et al. 1995; Heiskari et al., in press), and this protein product, although present in the kidney, does not seem to be expressed in the glomerular basement membrane (Peissel et al. 1995).

We also included in this study families in which the diagnosis of AS was either possible or doubtful and without clear-cut X-linked inheritance to verify the power of molecular testing, with respect to clinical diagnosis. Thus, we included sporadic and even autosomal cases, since in small families autosomal inheritance cannot be easily distinguished from X-linked (table 3). The finding of several COL4A5 mutations in patients with "possible" or even "doubtful" AS diagnosis, although at a lower rate (29% and 11%, respectively), indicates a higher specificity of molecular diagnosis over more traditional clinical and histological criteria. An example of this concept is represented by patient CNT, a 14-year-old female with negative family history. She underwent molecular analysis solely on the basis of hematuria and a renal biopsy strongly doubtful for AS. A de novo in-frame deletion of three amino acids was identified in her DNA. Molecular testing is even more powerful in relatives of patients in whom mutations have been identified. The mother of female patient FER was thought to be a carrier of AS, on the basis of sensorineural hypoacusis affecting high and low tones and posterior lenticonus. Molecular analysis excluded the presence of the ΔS942 deletion detected in her daughter, which indicates that the mutation arose de novo and that her ocular and ear signs are not related to AS. Thus, while anterior lenticonus can be specific of AS, posterior is not, in agreement with previous reports (Govan 1983).

The overall number of different mutations identified in this group of patients was 57. Mutations are scattered throughout the entire coding region and involve 31 of the 51 exons (fig. 1). Only five of these mutations were previously reported in other patients (Knebelmann et al. 1992; Lemmink et al. 1993; Boye et al. 1995; Heiskari et al., in press). The absence of a common mutation in AS precludes the use of efficient diagnostic methods based on the detection of known mutations (Forrest et al. 1995). Therefore, systematic scanning for mutations in the entire coding region, using methods such as SSCP

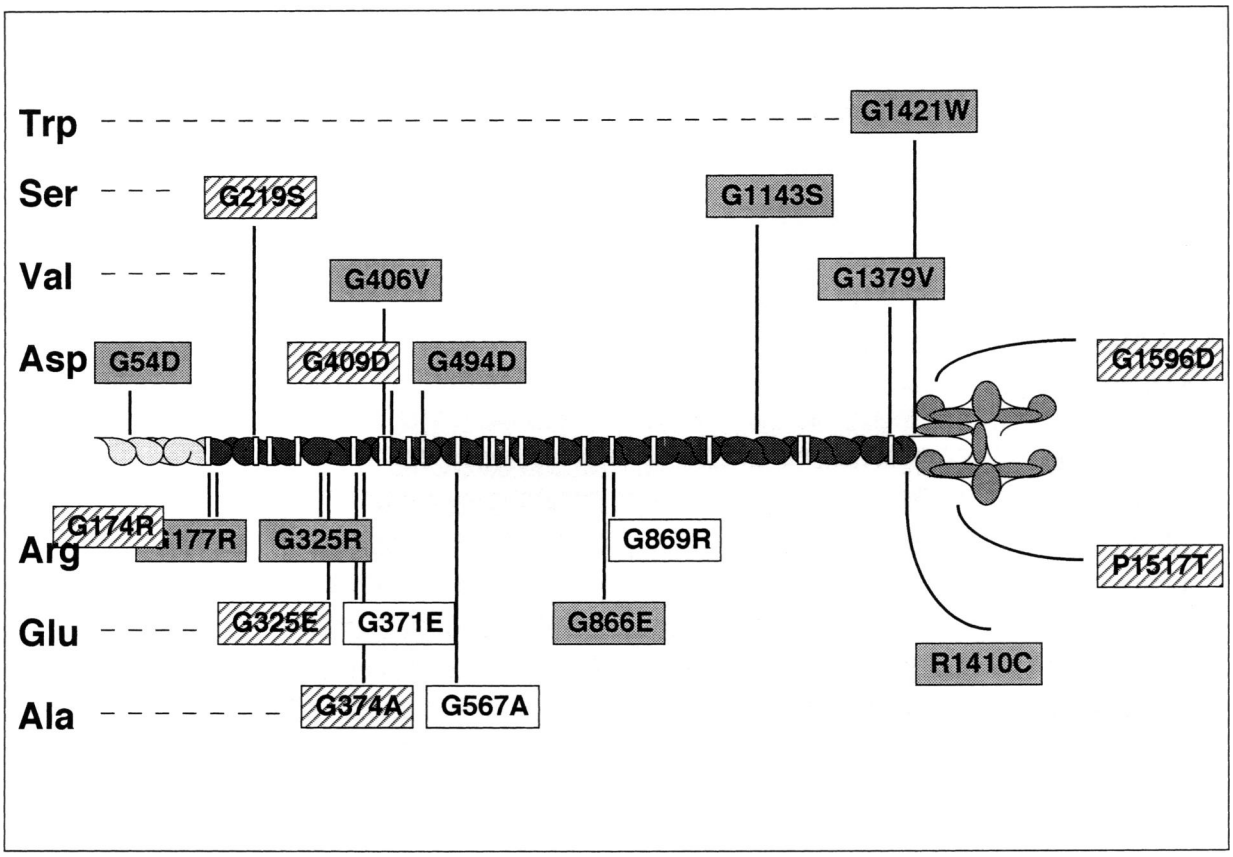

Figure 2 Missense mutations. Distribution of missense mutations along the various α5 chain domains: 7S (light gray), triple helix (dark gray) with characteristic interruptions (white bars), and the globular NC domain (light gray). Mutations are named according to the suggested nomenclature (Beaudet and Tsui 1993). Glycine substitutions are associated with a variable age at onset of ESRD in males: adult type (gray boxes), juvenile type (white boxes), and not determined, such as in children (striped boxes). Identical substituting amino acids for glycine are aligned in the same row. Note that gly changes to trp, ser, val, and asp are found in adult type (*top*), while gly-to-arg, -glu, and -ala changes are found in either juvenile or adult phenotype (*bottom*). Mutations G1379V and G494D were assigned to adult type on the basis of the maternal grandfathers' age at onset of ESRD (53 and 46 years, respectively). Among mutations in the triple helical domain, R1410C is the only one that does not involve glycine; this mutation creates an additional cysteine at the end of triple helix, which may interfere with disulfide bond arrangement in the adjacent NC domain. Mutations G1596D and P1517T are the only two substitutions in the NC domain.

analysis, is the elective strategy (Renieri and De Marchi, in press).

Altogether, we identified in the COL4A5 gene 31 single-base substitutions, 16 small deletions, and 6 insertions. Substitutions included 20 missense, 1 start codon, 1 nonsense, 4 splice site, 4 silent, and 1 intronic mutation. Five of these occur within a CpG dinucleotide changing a CG to a TG in two cases and CG to a CA in the others. Fifteen substitutions affect the G belonging to a GG dinucleotide, which represents the most mutable dinucleotide after CG. The 16 small deletions included 11 frameshift, 4 in-frame and 1 splice-site mutation. Six are deletions of >1 bp, and each of these falls in a region containing short direct repeats. These deletions fit with the model of slipped mispairing, in which one copy of the repeat is lost along with the intervening sequence (Krawezak and Cooper 1991).

The clinical and molecular analysis of a large series of carefully described patients such as the one collected in the present study (60 families with 57 different mutations) allows one to draw significant genotype-phenotype correlations. Detected mutations can be predicted to affect α5(IV) chain synthesis or structure in a variety of ways (figs. 1 and 2 and table 2). This might in part explain the variable outcome of AS, which shows an heterogeneous phenotype at both the clinical and histological level (Habib et al. 1982; Feingold et al. 1985; Gubler et al. 1993). Before the advent of molecular diagnosis, the syndrome was split into six different forms, purely on the basis of clinical examination and of formal genetic analysis (Hasstedt et al. 1986). This subdivision, in particular the distinction between juvenile and adult AS, appears to correlate with the type of mutations observed (table 4): missense mutations are significantly associated with onset of ESRD at >31 years in males (Fisher's exact test $P = .0026$). If similar comparison is performed among females, no significant association between mutation type and presence or absence of ESRD

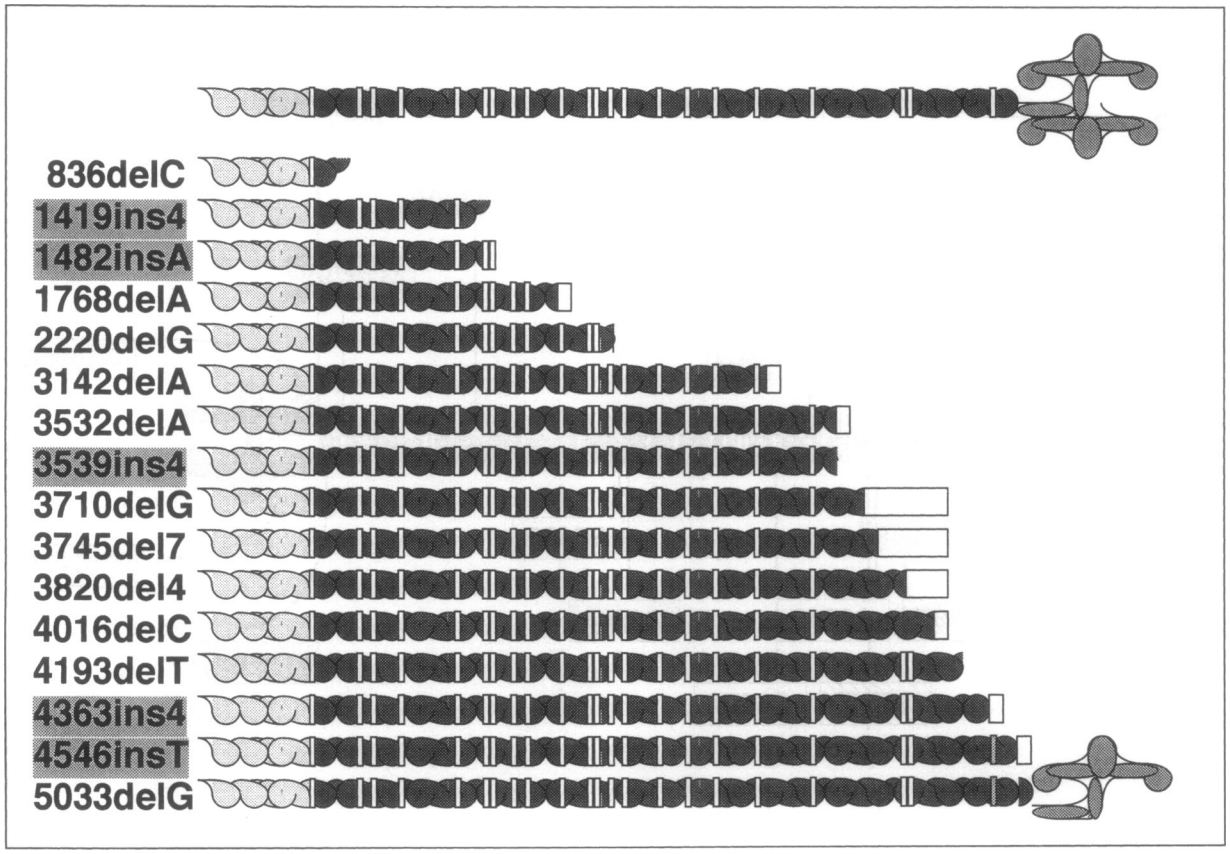

Figure 3 Frameshift mutations. Predicted truncated proteins resulting from the various frameshift mutations are shown. Domains of the protein are represented, as in figure 2. Ending white boxes are out-of-frame polypeptide stretches. Mutations are named with the cDNA position followed by the deleted (del) or inserted (ins) single nucleotide, or the numbers of nucleotides. Insertions are shown as gray boxes. The smallest protein contains the 7S domain only, the largest contains the entire collagenous region and one of the two symmetrical halves of the NC domain. Four different mutations (3710delG, 3745del7, 3820del4, and 4016delC) in three different exons lead to the same frameshift.

is observed (P = .8433; data not shown). Thus, other factors seem to be responsible for variable expression in females (such as X-inactivation pattern), while in males null mutations in the COL4A5 gene (major rearrangements and frameshifts) cause juvenile-type AS (Renieri et al. 1993, 1995), and the adult type of AS appears to be limited to some missense mutations that affect glycine residues (table 4 and fig. 1). The severity of the phenotype is likely to depend both on the type and the position of substituting amino acid. This situation is reminiscent

Table 3

COL4A5 Mutations in Families with Different Diagnostic Scores and Modes of Inheritance

	Diagnostic Score							
	Certain/Likely		Possible		Doubtful		Total	
Inheritance	No. Families	Mutation Present (%)	No. Families	Mutation Present (%)	No. Families	Mutation Present (%)	No. Families	Muation Present (%)
X-linked	96	43 (45)	24	7 (29)	9	1 (11)	129	51 (40)
Sporadic	18	3 (17)	7	2 (28)	12	1 (8)	37	6 (16)
Not applicable	5	1 (20)	2	1 (50)	3	1 (33)	10	3 (30)
Autosomal recessive	9	0 (0)	6	0 (0)	4	0 (0)	19	0 (0)
Autosomal dominant	2	0 (0)	3	0 (0)	1	0 (0)	6	0 (0)
Total	130	47	42	10	29	3	201	60

Table 4

Correlation of Mutations with Phenotypes in Males

Age at Onset of ESRD (years)	Major Rearrangement	Small Mutations						Total
		Missense[a]	Frameshift	Stop	Splice Site	In Frame	Start	
ESRD <31	7	5	8	1	2	1	1	25
ESRD >31	...	9	...	...	...	...	...	9
No Data	2	9	8	...	2	4	...	25
Total	9	23	16	1	4	5	1	59

[a] Missense versus all other mutations $P = .0026$.

of the case of osteogenesis imperfecta, in which the two components have first been recognized (Byers 1990) and for which a regional model relating the mutation site with the phenotype has been proposed (Wang et al. 1993). In conclusion, our findings demonstrate that direct mutation diagnosis of AS is feasible and specific, allow to estimate its sensitivity, and help in the design of a strategy for mutation identification in X-linked AS. Moreover, these observations have important implications in the diagnosis, prognosis and follow-up of patients with AS and in the genetic counseling of families with this disorder.

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