X-linked Alport Syndrome: Natural History in 195 Families and Genotype- Phenotype Correlations in Males

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Abstract. Alport syndrome (AS) is a type IV collagen hereditary disease characterized by the association of progressive hematuric nephritis, hearing loss, and, frequently, ocular changes. Mutations in the COL4A5 collagen gene are responsible for the more common X-linked dominant form of the disease. Considerable allelic heterogeneity has been observed. A "European Community Alport Syndrome Concerted Action" has been established to delineate accurately the AS phenotype and to determine genotype-phenotype correlations in a large number of families. Data concerning 329 families, 250 of them with an X-linked transmission, were collected. Characteristics of the 401 male patients belonging to the 195 families with COL4A5 mutation are presented. All male patients were hematuric, and the rate of progression to end-stage renal failure and deafness was mutation-dependent. Large deletions, nonsense mutations, or small mutations changing the reading frame conferred to affected male patients a 90% probability of developing end-stage renal failure before 30 yr of age, whereas the same risk was of 50 and 70%, respectively, in patients with missense or splice site mutation. The risk of developing hearing loss before 30 yr of age was approximately 60% in patients with missense mutations, contrary to 90% for the other types of mutations. The natural history of X-linked AS and correlations with COL4A5 mutations have been established in a large cohort of male patients. These data could be used for further evaluation of therapeutic approaches.

Alport Syndrome (AS) is characterized by progressive hematuric nephritis with ultrastructural and immunohistochemical changes of the glomerular basement membrane (GBM), frequently associated with sensorineural hearing loss (1–8). All affected male patients progress to renal failure, whereas in most female patients the course is considered to be benign. The estimated prevalence of the disease in the United States is 1:5000 (2). It accounts for 1 to 2% of patients reaching end-stage renal disease (ESRD) in Europe (9) and 2.3% of the transplant population in the United States (2). These figures are probably an underestimate, as the diagnosis may be missed when it occurs in small families or sporadically. AS is clinically heterogeneous, and numerous phenotypes have been described according to the rate of progression to ESRD, the type of GBM changes, the presence or absence of deafness, and other extrarenal manifestations such as ocular changes, macrothrombocytopenia, and diffuse esophageal leiomyomatosis. Genetic heterogeneity has been demonstrated as well (10).

AS is caused by defects in type IV collagen, a major structural component of basement membranes. Six type IV collagen genes have been cloned and characterized, and are localized in pairs on three chromosomes (11). The COL4A3 and COL4A4

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genes, on chromosome 2, are involved in the rarer autosomal recessive forms of the disease (12–14), whereas mutations in the COL4A5 gene coding for the α5 chain of type IV collagen are responsible for the more frequent X-linked dominant form of AS (15,16). The COL4A5 gene comprises about 250 kb of genomic DNA and contains 51 exons encoding a 6.5-kb transcript (17). The α5 chain contains 1685 amino acids, divided into a 26-residue signal peptide, a 1430-residue collagenous domain containing short noncollagenous interruptions, and a 229-residue carboxy-terminal noncollagenous (NC) domain (18). Since the first COL4A5 mutations were described by Barker *et al.* (19), nearly 300 mutations have been reported by different groups in Europe, the United States, and Japan (reviewed in references (16) and (20) through (24). Considerable allelic heterogeneity has been observed, necessitating the analysis of a large number of AS families to look for genotype-phenotype correlations.

We established a "European Community Alport Syndrome Concerted Action" (ECASCA) focused on: (*1*) the accurate delineation of the AS phenotype in a large number of patients/families; (*2*) the generation of a database; and (*3*) the identification of genotype-phenotype correlations. Priority was given to the collection data from families with an identified mutation. This program enabled the collection of information on 329 AS families, which is the largest series in the world. The disease was X-linked in 250 families (76%) and in 195 of them a COL4A5 mutation had been identified. Here, we analyze the clinical and pathologic characteristics of these 195 families and correlate phenotype with genotype in male patients.

Materials and Methods

Criteria for Inclusion in the Study

The ECASCA study (1994–1997) was conducted by 12 groups in 12 different European countries, approved by ethics committees locally. Criteria for inclusion of families were based on the classical criteria of AS, namely, positive family history of hematuria with or without progression to ESRD, progressive sensorineural hearing loss, characteristic ocular changes (lenticonus and/or maculopathy), and typical ultrastructural changes of the GBM. Two additional signs, diffuse esophageal leiomyomatosis (25) and abnormal GBM distribution of the α3, α4, and α5 chains of type IV collagen (3,8,26), were considered as new diagnostic criteria. The detection of COL4A5, COL4A3, or COL4A4 mutations was decisive for the diagnosis of the disease whatever the number of clinical or morphologic criteria.

Patient Database

A questionnaire (available on request) was developed to standardize the information. National coordinators were responsible for the distribution of the questionnaires and the collection of data. Data were anonymous; each family and each patient were given a code comprising the nationality and a number. The curator of the database had no information on personal data, except the birth date and family tree. The database is accessible only to participants of the Concerted Action, who agreed in writing to guarantee the confidentiality of the information. The questionnaire consisted of two parts. The first gathered information at the family level: pedigree, familial expression of the disease, and the result of a mutation search. The second part consisted of an individual questionnaire.

Currently, questionnaires concerning 329 families with 1234 patients have been returned and entered into the database. These families were located in Belgium (19), Denmark (7), Finland (8), France (135), Germany (46), Holland (6), Italy (56), Poland (5), Portugal (4), Spain (4), Sweden (17), and the United Kingdom (22). The disease was X-linked in 250 families (76%), with identified COL4A5 mutation in 195, most of them having been published previously by the members of the cooperative group (reviewed in references (16), (18), (20), (21), (23), and (24). The mode of transmission was autosomal recessive in 26 families (7.90%), autosomal dominant in two, and could not be determined in 51 because of the small size of the family, and the failure to detect mutation.

Statistical Analyses

The database was developed with ORACLE 6.09 software. Statistical analyses were done using SAS 6.09 and S+3.3 packages. Graphs of the occurrence of events (age at ESRD, age at detection of hearing loss) were computed according to the Kaplan–Meier method. For the comparison between graphs, robust statistical tests were used, taking into account the intrafamilial correlations (27).

Results

Expression of the Disease at the Family Level (195 Families)

Expression of the disease at the family level is presented in Table 1. Relevant findings are: (*1*) the absence of family history in 22 patients with COL4A5 mutations, showing that the incidence of *de novo* mutations in this series is approximately 12%; (*2*) the absence of hematuria in two families; these two families were unique in that only one female patient with diffuse leiomyomatosis and no renal symptom was affected in each one, the diagnosis of AS being ascertained by the detection of a *de novo* mutation; hematuria was a constant feature in all other families; and (*3*) the absence of ultrastructural changes of the GBM in two families in which only one nonhematuric female patient affected with diffuse esophageal leiomyomatosis has been examined. Interestingly, 38% of

Table 1. Summary of clinical and pathologic findings in the 195 Alport syndrome families with proven COL4A5 mutation[a]

Variable	Families with Proven COL4A5 Mutation
Consanguinity	5 of 192 (2.5%)
Familial history	171 of 193 (88.5%)
Hematuria	191 of 193 (99%)
ESRD	146 of 193 (76%)
Hearing loss	156 of 189 (82.5%)
Ocular changes	66 of 149 (44%)
Leiomyomatosis	9 of 176 (5%)
Ultrastructural GBM changes	115 of 117 (98%)
Immunohistochemical GBM changes	23 of 27 (85%)
Transplantation	106 of 195 (54%)
Posttransplantation anti-GBM GN	3 of 80 (4%)

[a] ESRD, end-stage renal disease; GBM, glomerular basement membrane; GN, glomerulonephritis.

COL4A5 mutations were detected in families presenting fewer than three diagnostic criteria for Alport syndrome (Table 2).

Expression of the Disease in Males (401 Patients)

Presenting clinical manifestation, known in 218 male patients, consisted of microscopic or macroscopic hematuria (isolated or associated with proteinuria) in 81%, proteinuria in 12.5%, and more severe symptoms (deafness, chronic renal failure, and/or hypertension) in the others. All patients had microscopic hematuria during the course of the disease, and a single or recurrent episode of gross hematuria occurred in 62%. Proteinuria was found in 95% of patients. Moderate hypertension was a variable and usually found late. Clinical hypoacousia, or hearing loss detected by systematic audiometry (bilateral hearing deficit in the 2000 to 8000 Hz range), developed in 79% of the 303 patients tested. Specific ocular changes were found in 35.2% of the 162 patients examined. They consisted of lenticonus (13%), maculopathy (13.6%), or the association of both lesions (8.6%). In addition, nine patients presented with congenital or early onset cataract. Progression to chronic or ESRD occurred in 282 (78.3%) of the 360 patients with follow-up information. The age at ESRD was known in 233 of 251 patients: it was $<$31 yr in 76.5% of patients (and $<$20 yr in 51% of them), between 31 and 40 yr in 17.5% of patients, and $>$41 yr in 6%. The median renal survival rate was 25 yr. Ninety percent of patients had reached ESRD before the age of 42. As shown in Figure 1, the risk of developing ESRD before the age of 30 or 40 is 70 and 90%, respectively, and the risk of developing deafness before the age of 40 is about 90% in these patients. At the last examination, most patients with ESRD were alive and treated by hemodialysis or renal transplantation.

An ultrastructural examination of the GBM was performed in 98 patients at a mean age of 12.4 yr. Thickening, with or without segmental thinning, of the GBM was observed in 88% of patients (mean age 12.5 yr), and diffuse thinning alone in 12% (mean age 11 yr). Immunohistochemical study of the GBM revealed the absence of the α3(IV) chain (and of the α4 (IV) and α5 (IV) chains in the nine patients studied) in 14 of the 16 examined patients and the normal expression of the three chains in two.

Table 2. Number of diagnostic criteria in 193 Alport syndrome families with COL4A5 mutations

No. of Criteria[a]	No. of Families	Percentage of Families
0	1	0.5%
1	14	7.5%
2	58	30%
3	79	41%
4	31	16%
5	9	4.5%
6	1	0.5%

[a] In addition to the presence of hematuria in the proband. Two unrelated nonhematuric female patients with sporadic and isolated diffuse esophageal leiomyomatosis were excluded from the analysis. The following diagnostic criteria were scored: family history of hematuria; progressive sensorineural hearing loss; characteristic ocular changes (lenticonus and/or maculopathy); typical ultrastructural changes of the GBM; diffuse esophageal leiomyomatosis; and abnormal GBM distribution of the α(IV) chains.

COL4A5 and COL4A5/COL4A6 Mutations

COL4A5 mutations identified in the 195 families consisted of a large deletion of the COL4A5 gene in 19.5% (also involving the COL4A6 gene in 6%) and a small mutation in the others (Table 3).

Large COL4A5 Rearrangements: 38. The size of the 38 deletions varied from the absence of single or a few exons to the complete absence of the gene in two kindreds. Intragenic deletions were distributed all along the gene. Twelve deletions removed the 5′ end of both COL4A5 and the contiguous COL4A6 genes; in nine of them the breakpoint was located in the second intron of COL4A6.

Other COL4A5 Mutations: 157. The 157 other COL4A5 mutations were distributed all along the gene and consisted of:

- *Deletions* (31), involving one or a few base pairs and resulting in frameshift at the mRNA level in 27 families or leading to a stop codon in one. In three families, the deletion was in frame and responsible for the loss of one to six codons; they were analyzed with missense mutations.
- *Insertions* (9), consisting of the addition of 1 bp in most patients, responsible for an mRNA frameshift in eight families and for the occurrence of a stop codon in one. All insertions were predicted to produce a premature stop codon leading to a truncated α5(IV) chain.
- *Missense mutations* (59), characterized by guanine substitutions in the first or second position of glycine codons and leading to glycine substitution in the collagenous domain of the α5(IV) chain, were observed in 58 families (two glycine substitutions were detected in one family) and represented the most frequent type of mutation detected in our series (30% of mutations).
- *Other missense mutations* (16) leading to various amino acid substitutions in the collagenous (8) or the NC domain (8) of the chain. Interestingly, two of these mutations converted highly conserved cysteine residues of the NC domain to arginine or tyrosine, respectively.
- *Nonsense mutations* (12). Single base substitutions leading to premature stop codons, were detected in 14 kindreds. Nine mutations (one of them observed in two unrelated families) occurred in the collagenous domain and are predicted to produce a truncated α5(IV) chain with no NC1 domain. Three mutations (one of them observed in two unrelated kindreds) occurred in exons 48 (2) or 51 (1) in the NC1 domain, which would result in an α5(IV) chain with a truncated NC1 domain.
- *Splice-site mutations* (29). One base pair deletion in two kindreds and single base substitutions in 27 were found at consensus splice sites, in the collagenous domain of COL4A5 in 24 families and in the NC1 domain in five.

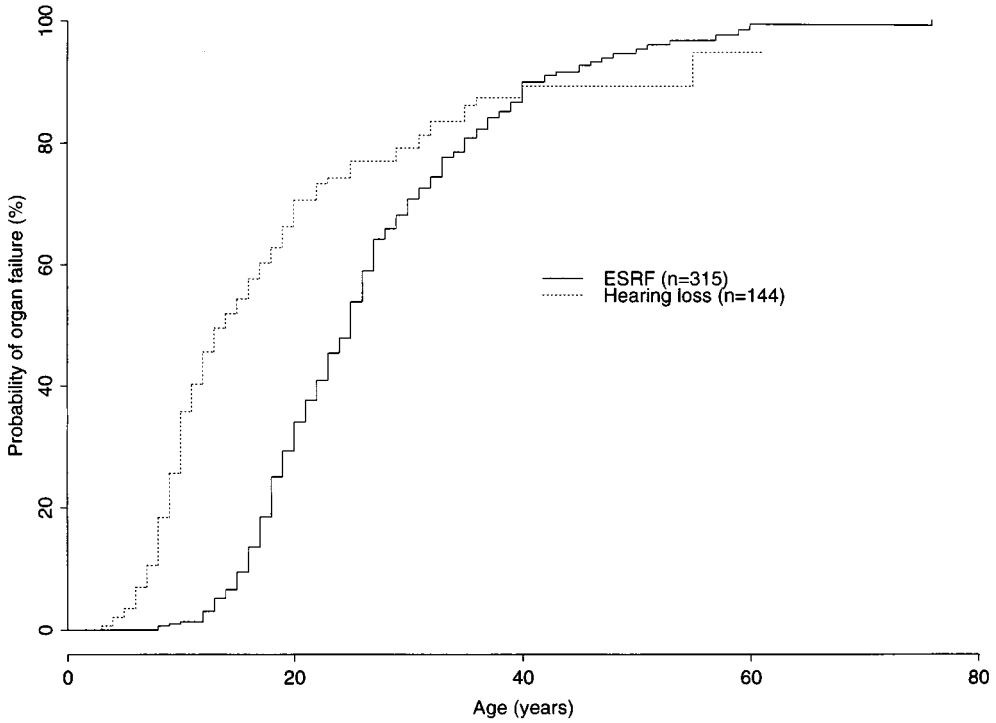

Figure 1. Probability of end-stage renal disease (ESRD) or hearing loss in male patients with COL4A5 mutation. Precise chronological data for evolution of renal and auditory functions were obtained in 315 and 144 patients, respectively.

Table 3. Mutations in 195 kindreds[a]

Type of Mutation	No. of Families	Percentage of Mutations
Large rearrangement		
COL4A5 deletion	26	13.5
COL4A5+A6 deletion	12	6
Small mutation		
missense	74	37.95
deletion[b]	31	15.9
insertion[c]	9	4.6
nonsense	14	7.2
splice site	29	14.9

[a] Two missense mutations leading to glycine substitution were found in one family. One nonsense mutation was present in two unrelated families.
[b] Twenty-eight are frameshift and three are in-frame deletions.
[c] All insertions are frameshift mutations.

These mutations were expected to lead to the deletion of one or several COL4A5 exons.

Correlations between Genotypes and Phenotypes

Progression to ESRD. The probability of developing ESRD before the age of 30 is greater than 90% in male patients with large rearrangement of COL4A5 or with small mutations leading to premature stop codons (Figure 2). No significant differences with respect to age at ESRD were found between these two groups, or between the different types of small mutations (frameshift deletions, insertions, nonsense substitution) (data not shown). The overall rate of progression appeared to be slower in male patients with splice site or missense mutations, with a probability of having developed ESRD at 30 yr in 70 and 50% of patients, respectively. Differences between 1 = large rearrangements + small mutations leading to premature stop codons, 2 = splice site mutations, and 3 = missense mutations were highly significant ($P < 0.001$).

Intrafamilial correlation for the age of male patients at ESRD could be established in 23 kindreds in which at least three (between 3 and 11) male patients have progressed to ESRD (Figure 3). Intrafamilial homogeneity (<15 [1 to 14]-yr difference in the age at ESRD) was observed in 17 kindreds. However, in one of these families (family 1) in which five male patients developed ESRD between 17 and 26 yr of age, one patient had a more severe course and reached ESRD at 9 yr. Most of these 17 families had large rearrangement of COL4A5 or small mutation leading to stop codon, and four had missense and two splice site mutations. Conversely, intrafamilial heterogeneity (>20 [20 to 56]-yr difference in the age at ESRD) was observed in six kindreds with frameshift (one family), splice site (two families), or missense (three families) mutations.

Occurrence of Hearing Loss. The probability of developing hearing loss before the age of 30 in male patients was also affected by the type of COL4A5 mutation (Figure 3). The risk was about 60% in patients with a missense mutation, whereas it reached 90% for the other types of mutations together including splice site mutations ($P < 0.005$). Interestingly, hearing could remain normal over the age of 50 in some

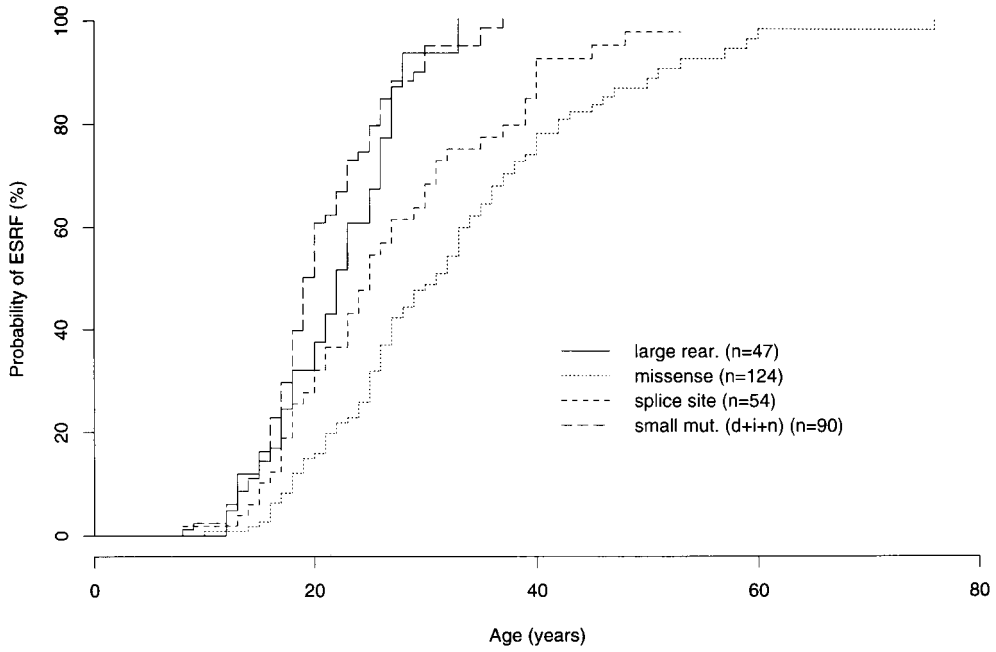

Figure 2. Probability of ESRD in 315 male patients according to the type of COL4A5 mutation. rear., rearrangement; mut., mutation; d, deletion; i, insertion; n, nonsense mutation. Three groups (1 = large rearrangements + small mutations leading to premature stop codons; 2 = splice site mutations; 3 = missense mutations and three small in-frame deletions) were defined for statistical comparison. Differences between group 1 *versus* group 2, group 1 *versus* 3, and group 2 *versus* group 3 were highly significant ($P < 0.001$).

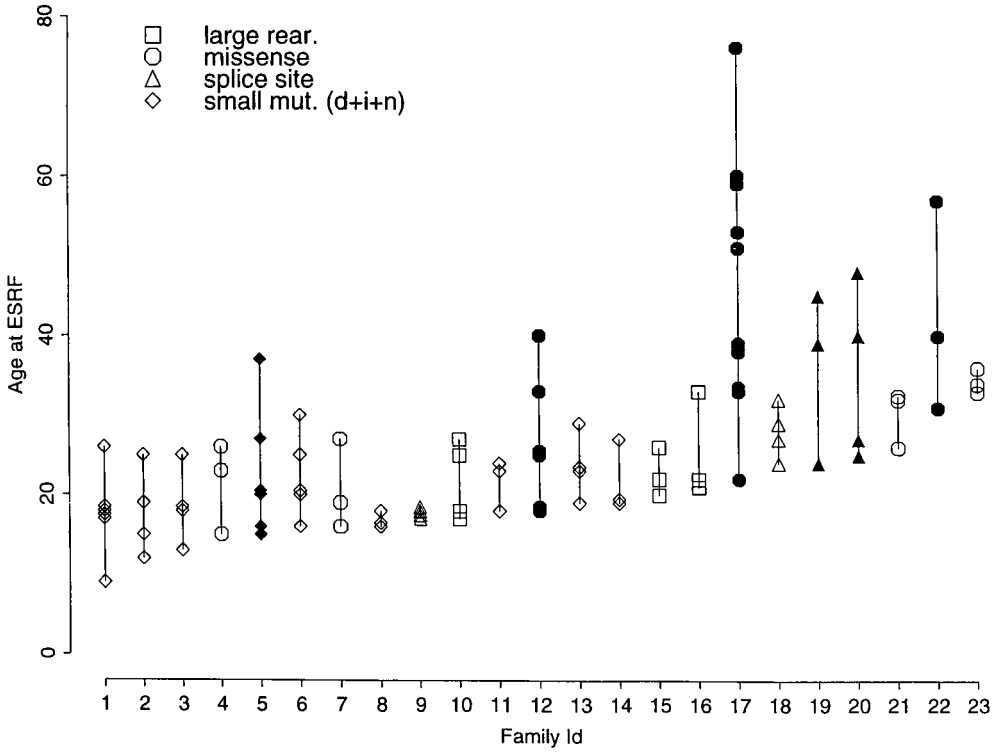

Figure 3. Age of male patients at ESRD in the 23 families in which at least three male patients have progressed to ESRD showing intrafamilial homogeneity in 17 kindreds (open symbols), *versus* large variability in six families (filled symbols). The 23 families have been ordered according to increasing age at ESRD for the most severely affected proband. Abbreviations as in Figure 2.

male patients with a missense mutation. No significant differences were observed between the diverse small mutations changing the reading frame; all 15 patients with nonsense mutations developed hearing loss before the age of 20.

Occurrence of Ocular Lesions. Lenticonus was found in 35 patients. It was more frequent in patients with a large COL4A5 deletion or small mutation leading to premature stop codon than in patients with missense or splice site mutations (Table 4). The difference between groups was highly significant ($P < 0.001$). Early progression to ESRD was observed in the 27 families with large deletion or small mutation leading to premature stop codon. In one family with glycine substitution, no progression to chronic renal failure has been observed in the only affected male of the kindred, age 30 at last examination. In the other families, early progression to ESRD was observed in affected male patients, at ages 16, 22, and 31 in patients with missense mutation, and at ages 8, 16, 18, 21, 23, 23, and 30 in patients with splice site mutation. Maculopathy was observed in 36 patients. No significant correlation with the type of mutation was observed. Congenital or early onset cataract found in nine patients was associated with diffuse esophageal leiomyomatosis (DEL) and large COL4A5-COL4A6 deletion in seven of them.

Diffuse Esophageal Leiomyomatosis. In all nine cases of DEL, a deletion removing the 5′ end of both the COL4A5 and the COL4A6 genes, with a breakpoint located in the second intron of COL4A6, was identified. DEL was not present in the three kindreds with a COL4A6 deletion extending beyond the second intron, and was never observed among patients with a mutation restricted to the COL4A5 gene.

Ultrastructural and Immunohistochemical Changes of the GBM. As seen in Table 5, all patients with large deletion of COL4A5 had GBM thickening. Patients with another type of mutation had either thick, thick and thin, or diffusely thin GBM. GBM staining for α3 (IV) (and for α4 (IV) and α5 (IV) in the nine patients tested) was lacking in 14 male patients with different types of COL4A5 mutations (5 large deletions, 4 missense, 1 splice site, and 4 small mutations [nonsense, deletion, or insertion changing the reading frame]). Conversely, GBM expression of the three chains was normal in two patients, one with glycine substitution and one with splice site mutation.

Table 4. X-linked Alport syndrome: correlation between the type of mutation and the occurrence of lenticonus

Mutation	Lenticonus		
	Absent	Present	Frequency (%)
Large rearrangement	21	11	34.40
Nonsense deletion insertion	28	16	36.40
Splice site	20	4	16.7
Missense	58	4	6.45
Total	127	35	21.60

Table 5. X-linked Alport syndrome: correlation between the type of COL4A5 mutation and the type of GBM ultrastructural changes in 93 male patients

GBM	Mutation			
	Large Rearrangement	Missense	Splice Site	Nonsense Deletion Insertion
Thin (12 pts)	0	7	1	4
Thick and thin (57 pts)	13	24	6	14
Thick (24 pts)	7	9	1	7

Renal Transplantation. Transplantation was performed in 118 male patients. Three unrelated patients (2.5% of transplanted male patients) developed anti-GBM glomerulonephritis leading to rapid graft loss. All three patients had a large deletion of the COL4A5 gene. Interestingly, 16 other patients with a large rearrangement of COL4A5 and 32 with a small mutation who also were expected to produce a truncated α5(IV) protein lacking the NC domain did not develop anti-GBM glomerulonephritis in the graft.

Discussion

The ECASCA multicentric study allowed the analysis of 329 AS families. Criteria for inclusion were based on the classic signs of the disease: familial hematuria with or without progression to ESRD, sensorineural hearing loss, specific ocular lesions, and abnormal GBM ultrastructure. Two additional signs, diffuse esophageal leiomyomatosis (25) and abnormal GBM expression of the α3, α4, and α5 chains of type IV collagen, specifically observed in AS kindreds (3,8,26), were regarded as new diagnostic criteria.

The disease was X-linked in 250 families, and COL4A5 mutations were identified in 195, 38% having less than three diagnostic criteria for the disease. The analysis of the phenotypic expression of the disease in male patients included in this series, the largest studied to date, brings precise information on the natural history of the disease. Based on this analysis, valuable genotype-phenotype correlations and evaluation of the actuarial risk of developing ESRD or deafness could be established.

Hematuria, usually microscopic, was the presenting symptom in 81% of male patients and was present in all of them during the course of the disease. It was associated with proteinuria in 95% of patients. Hearing loss was present in 79% of male patients and typical ocular changes (lenticonus, macular fleck, or both) were found in 35.2% of patients examined, results that can be compared with a rate ranging from 30 to 72% in previously reported limited series (2–4). Given its specificity in AS, ocular examination should be more widely used in the investigation of hematuric patients. The frequency

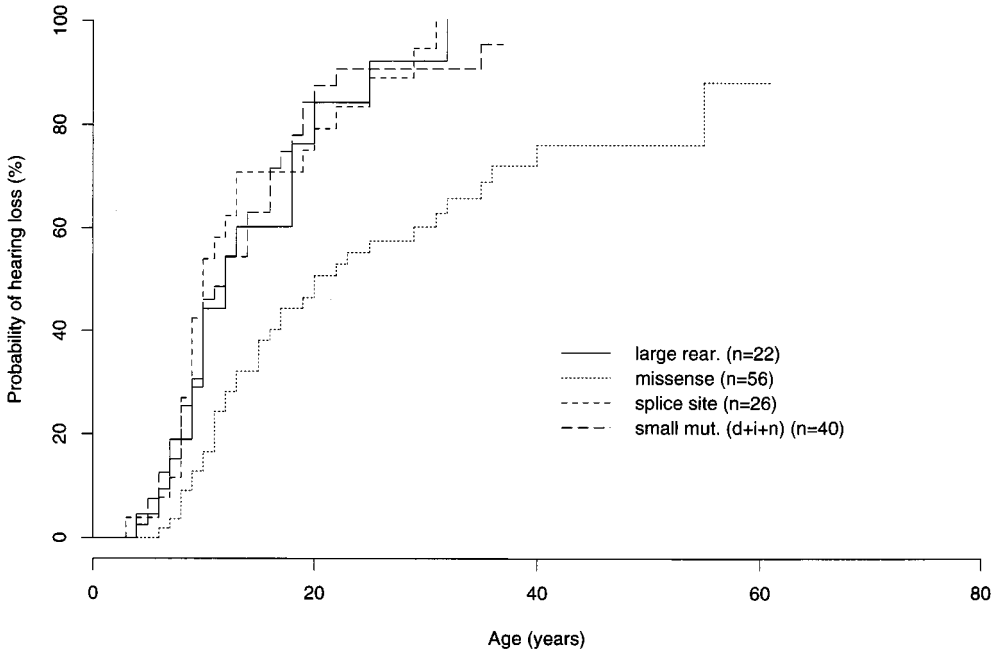

Figure 4. Probability of hearing loss in 144 male patients according to the type of COL4A5 mutation. Abbreviations as in Figure 2. Difference between patients with missense mutation and patients with other types of mutations were highly significant ($P < 0.005$).

of recurrent corneal erosion (28) has not been assessed in our study. Renal and auditory involvement are known to be severe in male patients and progression to ESRD is usually associated with deafness. We show that 90% of male patients are at risk of developing ESRD and deafness before the age of 40.

The most interesting aspect of this study is the opportunity to correlate genotype and phenotype in 195 families showing all kinds of COL4A5 mutations. We demonstrate the severity of large rearrangements of the COL4A5 gene, nonsense mutations, and deletion or insertion changing the reading frame of the gene, by showing that these mutations confer to affected male patients a probability of developing ESRD before the age of 30 of 90%, and a 50% renal survival rate of 20 yr. In contrast, the probability of developing ESRD before the age of 30 is 50% in patients with missense mutations, with a 50% renal survival rate of 32 yr. Intermediate severity of renal involvement is observed in patients with splice site mutations: The risk of developing ESRD before the age of 30 is 70%, with a 50% renal survival rate of 25 yr. Concerning hearing involvement, COL4A5 large deletions, nonsense mutations, small mutations changing the reading frame, and also splice site mutations confer a 50% risk of hearing defect at age 10, whereas missense mutations lead to the same risk at age 20. In some kindreds with missense mutations, both hearing loss and ESRD develop after the age of 50. This was also found in the large family reported by Barker *et al.* (29) with an arginine to leucine substitution in the COL4A5 noncollagenous domain. Variable severity of renal involvement was observed in patients with splice site mutations, altering or not altering the reading frame. RNA analysis may accurately determine their consequences (14,30). Intrafamilial homogeneity for the age of male patients at ESRD was observed in families presenting large rearrangement or frameshift mutation of COL4A5. Conversely, more than 20 yr difference in the age at ESRD was observed within five families with missense or splice site mutation. This variability, also described in three families with glycine or arginine substitution (29,31,32), has not been previously recorded in families with splice site mutations. Specific ocular changes were detected at first examination or during the course of the disease in one-third of patients. No significant correlation was observed between the finding of maculopathy and the type of mutation. Conversely, the frequency of lenticonus is significantly higher in patients with large COL4A5 deletion or small mutation resulting in premature stop codon. Moreover, lenticonus has an ominous prognostic significance: Except in a family with a single affected male who has not developed renal insufficiency at 30 yr of age, lenticonus was observed in families with early progression to ESRD whatever the type of mutation. In accord with our previous data (25), the combination of AS and DEL (and frequently cataract) was always associated with large deletions involving both COL4A5 and the two first exons of COL4A6.

Ultrastructural changes of the GBM were found in all male patients. Most of them had typical thick and split or, alternatively, thick and thin GBM, but in 12 male patients the GBM was diffusely thin. The finding of COL4A5 mutations in these patients demonstrates that “thin basement membrane” is not restricted to “benign familial hematuria.” GBM immunohistochemical staining for the α3(IV) chain (and the α4-α5(IV) chains in the nine patients tested) was negative in 14 of the 16 patients studied. This specific anomaly was observed in all patients presenting COL4A5 mutations leading to the absence or the synthesis of truncated α5 chains, but also in some patients with missense mutations. Conversely, normal GBM

incorporation of the defective α5 and the related α3 and α4 chains was observed in two patients, one with missense mutations and the other with splice site mutation. Thus, if the coabsence of the three α(IV) chains is an irrefutable marker of Alport syndrome, their normal GBM expression does not allow exclusion of the diagnosis.

Only three of the 118 transplanted male patients with identified mutation developed posttransplant anti-GBM glomerulonephritis. Interestingly, all three had a large COL4A5 deletion, confirming that the risk for these patients of developing anti-GBM glomerulonephritis reaches 15%, which represents a sixfold increase compared to the total AS population. However, anti-GBM glomerulonephritis did not occur in the majority of patients with the same type of mutation, or small mutations resulting in premature stop codon, showing that other factors are also implicated in the development of anti-GBM antibodies.

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