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Mutations in the *COL4A4* and *COL4A3* Genes Cause Familial Benign Hematuria

CÈLIA BADENAS,*† MANUEL PRAGA,‡ BÁRBARA TAZÓN,*† LAURENCE HEIDET,§ CHRISTELLE ARRONDEL,§ ANNA ARMENGOL,*† AMADO ANDRÉS,‡ ENRIQUE MORALES,‡ JUAN ANTONIO CAMACHO,¶ XOSE LENS,# SONIA DÁVILA,‡ MONTSE MILÀ,† CORINNE ANTIGNAC,§ ALEJANDRO DARNELL,* and ROSER TORRA*

**Nephrology and †Genetics Departments, Hospital Clínic, Barcelona, Spain; ‡Nephrology Department, Hospital 12 de Octubre, Madrid, Spain; §Nephrology Department, Inserm U423, Université René Descartes, Hôpital Necker-Enfants Malades, Paris, France; ¶Hospital Sant Joan de Déu, Barcelona, Spain; and #Nephrology Department, Hospital Clínico Universitario, Santiago de Compostela, Spain.*

Abstract. Familial benign hematuria (FBH) is a common autosomal dominant disorder characterized by the presence of persistent or recurrent hematuria. The clinical and pathologic features of this syndrome resemble those of early Alport syndrome (AS), and for this reason a common molecular defect has been proposed. The *COL4A3/4* genes seem to be involved in both autosomal AS and FBH. This study involves a linkage analysis for the *COL4A3/4* loci and a search for mutations within these genes in 11 biopsy-proven FBH families. Haplotype analysis showed that linkage to the *COL4A3/4* locus could not be excluded in eight of nine families. One family was not linked to this locus; however, it included three affected women who could be X-linked AS carriers. Two families were too small to perform linkage analysis. *COL4A3* and *COL4A4* mutation screening disclosed six new pathogenic mutations, two in the *COL4A3* gene (G985V and G1015E) and four in the *COL4A4* gene (3222insA, IVS23-1G>C, 31del11, and G960R). It is the first time that mutations within the *COL4A3* gene are described in families with FBH. This study clearly demonstrates the main role of the *COL4A4* and *COL4A3* genes in the pathogenesis of FBH.

Familial benign hematuria (FBH) (MIM 141200) is characterized by the presence of persistent or recurrent hematuria, usually detected in childhood. Hematuria remains isolated and, by definition, never progresses toward end-stage renal disease. Diffuse attenuation of the glomerular basement membrane (GBM) is usually considered the hallmark of the condition, but it is not specific. Due to this pathologic finding, the term, "thin basement membrane disease," is often used to designate FBH. However, this term is misleading because the finding of thin GBM is not the guarantee of a benign disease (1–3). The pattern of inheritance of FBH is autosomal-dominant, and the prognosis of the condition is excellent. However, the diagnosis of this benign disease may be difficult to establish because it is based on a series of negative findings (absence of proteinuria, renal failure, or extrarenal symptoms) and the finding of a nonspecific ultrastructural lesion, the thin GBM, and above all on the results of family investigations demonstrating the absence of progression toward renal failure. For these reasons, the diagnosis must be reconsidered if any new symptoms are observed during the follow-up period.

The major structural component of the GBM is a collagen IV network (4). The six α(IV) chains are encoded by six genes (*COL4A1-COL4A6*), which present a unique arrangement in that they are located pairwise in a head-to-head fashion on three separate chromosomes (4,5–12). The *COL4A5* gene accounts for 85% of cases (13–15), but the autosomal-recessive and -dominant Alport syndrome (AS) are due to mutations in the *COL4A3* and *COL4A4* genes, which are localized in the tip of the long arm of chromosome 2 (16–19). A mutation in the *COL4A3* gene has recently been described in a family with autosomal-dominant AS (18). This striking high molecular heterogeneity of the *COL4A3/4* genes has been enhanced since the description by Lemmnik *et al.* (20) of a large pedigree affected by AS and FBH. After this initial report, some authors have detected more mutations in the *COL4A4* gene in families with autosomal-recessive AS and hematuria in relatives (21–23). These findings showed that patients with FBH can be heterozygous for a mutation within the *COL4A3/4* genes. However, at least two groups recently showed that some FBH are not linked to this locus (24,25).

We performed linkage and mutation analyses in the

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Correspondence to: Dr. Roser Torra, Hereditary Renal Diseases, Nephrology Department, Fundació Puigvert, Cartagena 340-350, 08025 Barcelona, Spain. Phone: 34-93-4169700; Fax: 34-93-4169730; E-mail: rtorra@fundacio-puigvert.es
Present affiliation of Drs. Torra and Tazón: Fundació Puigvert, Barcelona, Spain.

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COL4A3/4 genes in 11 Spanish FBH families and detected four new mutations within the *COL4A4* gene and two new mutations in *COL4A3*.

Materials and Methods

Patients

Fifty-four subjects belonging to eleven unrelated families diagnosed with FBH were the object of the present molecular study. At least one member of each family had undergone a renal biopsy with ultrastructural examination and had a basement membrane thickness <264 nm. All families were Spanish. None of the subjects included in this study had either proteinuria or renal failure at the moment of performing the renal biopsy. The study was approved by the Ethical Committee of each hospital participating in the study.

The pedigrees are depicted in Figures 1 (families 1, 2, 4, to 11) and 2 (family 3). The group of affected subjects was composed of 12 men and 23 women with a mean age of 33.89 yr (SD, 17.7 yr; range, 10 to 76 yr). The age at the moment of detecting hematuria is shown beside each symbol in Figure 1. Thirteen patients underwent a renal biopsy, which is shown in Figure 1 with an arrow above each symbol. The mean follow-up period of these patients was 9 yr (SD, 4.5 yr; range, 2 to 18 yr). Four patients developed hypertension during the follow-up period (Figure 1). These patients are now 52, 52, 50, and 44 yr old respectively. No patient showed signs of neurosensorial deafness, but no audiogram was systematically performed. At the moment of diagnosis, none of the patients had proteinuria, but during the follow-up period, two patients, both belonging to family HFB-4, developed significant proteinuria. Patient I2 has a proteinuria of 1 g/24 h at 52 yr of age, and her son has a proteinuria of 2 g/24 h at 21 yr of age. At the moment of performing the renal biopsy, the patient had no proteinuria and the histologic findings were consistent with FBH. No further biopsies after the detection of proteinuria have been performed as yet.

In family HFB-3, the three affected women were 70, 46, and 20 yr old, respectively. None of them developed either hypertension or proteinuria after a follow-up period of 3, 13, and 12 yr, respectively.

None of the patients described in this article, even those with proteinuria, developed renal failure during the follow-up period.

Renal Biopsies

Renal biopsies were obtained percutaneously and processed for light, immunofluorescence, and electron microscopy. A Hitachi HU 12 A transmission electron microscope (Hitachi, Yokohama, Japan) was used. For morphometric analyses, a minimum of five capillary loops were studied. Ten transverse measures between endothelial and epithelial membranes, in areas separated from the mesangium, were performed in each loop. Areas with no sharp outlined membranes were discarded, assuming that they were on no orthogonal section. A thickness of 264 nm was considered as the cut-off point for the diagnosis of a thin GBM (26,27). FBH was diagnosed when the mean of the measures was below this point, and no images of thickening or splitting of GBM were observed.

Linkage Analyses

Blood samples from each individual were collected, and genomic DNA was extracted following the salting out procedure (28). The following markers were used: *D2S130*, *D2S360*, *D2S351*, *COL4A3-DNTR*, *D2S159*, *D2S401*, and *D2S396.* Family HFB-3, in which only women were affected, was analyzed with the following *COL4A5* markers: *DXS456*, *COL4A5–2B6*, *COL4A5–2B20*, and *DXS178*. One of the primers was fluorescently labeled. Amplification conditions were as described previously (16,19,29). The PCR products were analyzed on an ABI310 (Applied Biosystems, Foster City, CA).

Mutation Analyses

All exons of the *COL4A3* and *COL4A4* genes were screened for mutations in a biopsy-proven patient from each family. Exon sequences and primers for *COL4A4* were described by Boye *et al.* (22). Exon sequences and primers for the *COL4A3* gene were as in Heidet *et al.* (23).

The technique used to detect mutations was the single strand conformation analysis (SSCA). Three microliters of denatured PCR product were combined with loading buffer and loaded into GeneGel Excel 12.5 acrylamide gels (Amersham Pharmacia Biotech, Uppsala, Sweden) and run according to the manufacturer's instructions. The different migrations were silver-stained (30). DNA samples with abnormal migrations were sequenced. PCR products were purified using the QIAquick spin PCR purification kit (Qiagen, Chatsworth, CA) and automatically sequenced using the Dye Terminator Cycle Sequencing Ready Reaction (PerkinElmer Cetus, Norwalk, CT) and an ABI310 automatic sequencer (Applied Biosystems). The mutations described in this study were designated according to the recommendations of den Dunnen and Antonarakis (31).

Missense mutation G960R abolished a *Msp*I restriction site, and G985V created a *Rsa*I restriction site. These restriction sites were used to test for the presence of the mutations in relatives and controls. The digested PCR products were run on a 3% agarose gel and visualized by ultraviolet transillumination.

Results

Renal Biopsies

All biopsies disclosed a thickness of the GBM of <264 nm, and no images of thickening or splitting were observed. Mean GBM thickness in the present group of patients was 174 ± 19 nm. In addition, light microscopy and immunofluorescence were negative; in particular, no mesangial staining was observed with anti-IgA antibodies. The distribution of the type IV collagen chains has not been studied.

Linkage Analyses

Linkage analyses were performed with markers spanning the *COL4A3/4* locus in all families but two (HFB-4 and HFB-11), which were too small for being tested (Figure 1). In eight of these nine families, the results did not exclude linkage to the *COL4A3*/4 locus. Conversely, linkage to *COL4A3/4* was excluded in one family (Figure 2). This family included three affected women; therefore, linkage analysis to the *COL4A5* locus was performed to determine whether the three affected women could be X-linked AS carriers. Indeed, linkage analysis was compatible with this hypothesis (Figure 2).

Mutation Analyses

We detected six mutations, all of which are novel and private (Table 1). The three missense mutations were not found upon screening 200 control chromosomes.

Apart from the mutations described herein, we also identified several DNA changes that were polymorphisms or rare DNA variants (Table 2), which represented 81.2% (26 of 32) of all DNA changes detected in this study. We found heterozygous carriers of these variants among affected members of the

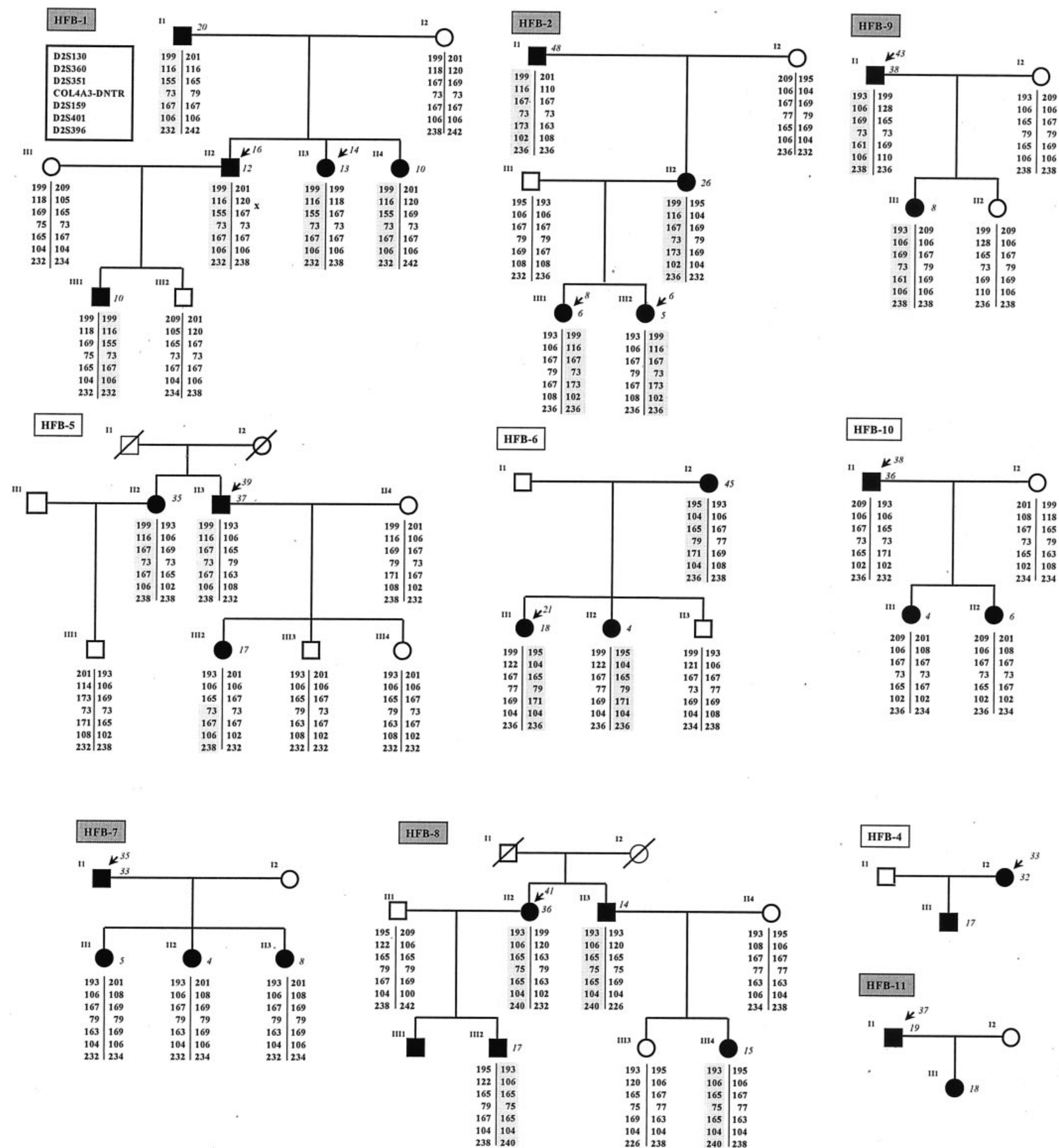

Figure 1. Familial benign hematuria (FBH) kindreds (except HFB-3). Affected individuals are indicated by the filled symbols, and unaffected individuals by unfilled symbols. Deceased individuals (diagonal lines) are of unknown phenotype. Below the symbol for each individual, genotypes for seven markers at *COL4A3/4* loci are shown in their chromosomal order, recombination events are marked with an X beside the haplotype. The identity of each marker is indicated in the box at the upper left. Inferred haplotypes shared by relatives with FBH are indicated by shadowed boxes. The arrows indicate the affected individuals with renal biopsy and the age when it was performed. The age when diagnosis was made is shown at the right of each affected individual. Families in which a mutation has been identified are indicated by shadowed boxes. Individuals in which high BP (HBP) has been detected are marked below each symbol.

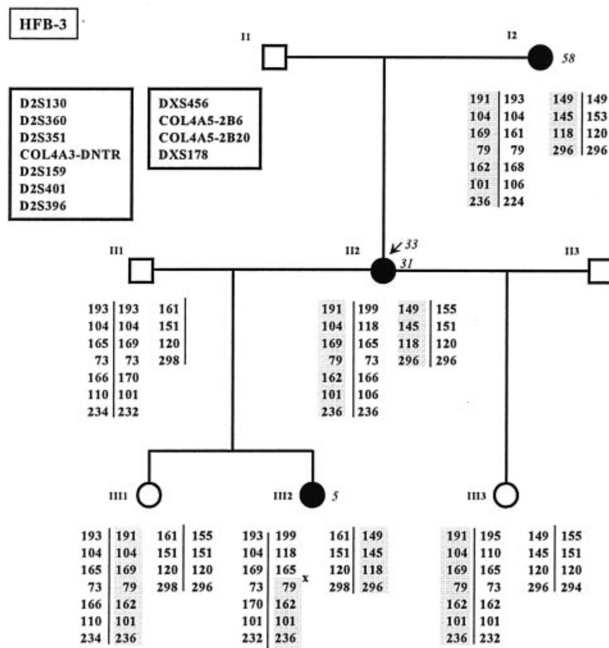

Figure 2. Family HFB-3 in which linkage analysis has been performed for the *COL4A3/4* and *COL4A5* loci. The identity of each marker is indicated in the box at the upper left. No common haplotype in shared by affected individuals in the *COL4A3/4* loci. On the other hand, haplotype of the X-chromosome is shared by the three affected women only.

families with no mutation detected, ruling out the possibility of large deletions involving most of these genes in these families.

Discussion

FBH was first described in 1966 as a dominant inherited persistent or recurrent microscopic hematuria not associated with other abnormalities such as renal failure or deafness (32). At early stages, AS can, however, be very similar to FBH, both in its initial clinical features and the electron-microscopic appearance. The characteristic finding in FBH is a diffuse thinning of the GBM; however, some lamellation has also been described (33,34). These pathologic features highly resemble those of early AS or Alport carriers.

Some mutations within the *COL4A4* and *COL4A3* genes have been described in families with autosomal-recessive AS where the heterozygotes present hematuria (20,22,23). Moreover Lemmink *et al.* (20) described a large family affected with autosomal-recessive AS, where patients found to be heterozygous for a G897E substitution in the *COL4A4* gene showed hematuria. However, neither are all families with FBH linked to the *COL4A3/4* locus (24,35), nor do all carriers of autosomal AS have hematuria. Thus, the consequences of the different mutations on the type IV collagen network are still not well understood.

In the present study, eight out of ten families are compatible with linkage to the *COL4A3/4* locus. In one family, linkage to the *COL4A3/4* locus was excluded. However, although the possibility of another autosomal locus for FBH cannot be ruled out, it is likely that X-linked AS is the underlying disease in this family with only affected women. As a mater of fact, families in which only women are classified as FBH may really be carriers of X-linked AS, a consideration that is crucial for genetic counseling. Hematuria can be due to other diseases; in contrast, hematuria in FBH can be intermittent, making the diagnosis very difficult. A precise diagnosis of FBH includes a renal biopsy, which is frequently omitted due to the benign nature of the disease. Moreover, in spite of having a renal biopsy compatible with FBH, it can correspond to another renal disease, which initially only presents as thin basement

Table 1. Description of mutations found in familial benign hematuria (FBH) families

Family	Mutation		Location	Predicted Effect	Restriction Site
	COL4A3	*COL4A4*			
HFB-1	G1015E		Exon 36	Missense mutation in the collagenous domain	
HFB-2	G985V		Exon 35	Missense mutation in the collagenous domain	*Rsa*I[a]
HFB-3					
HFB-4					
HFB-5					
HFB-6					
HFB-7		3222insA	Exon 35	Frameshift, stop after 1 amino acid	
HFB-8		IVS23-1G>C	Intron 23	Aberrant splicing of exon 24	
HFB-9		G960R	Exon 32	Missense mutation in the collagenous domain	*Msp*I[b]
HFB-10					
HFB-11		31del11	Exon 2	Frameshift, stop after 46 amino acids	

Restriction sites: [a]created or [b]abolished by mutations.

Table 2. Polymorphisms found in the *COL4A3/4* genes in this study

Exon	Nucleotide Change	Effect on Coding Sequence
COL4A3		
2	IVS2+12C>A	
	127G>C	G43R
9	473C>A	A158D
	485G>A	E162G
15	878C>G	P293R
17	976G>T	D326Y
21	1223G>A	R408H
	1195T>C	L399L
23	1452G>A	G484G
25	1721T>C	L574P
40	IVS39+18delA	
43	3807C>A	D1269E
48	4421T>C	L1474P
COL4A4		
2	−2C>T	
	17C>T	T6I
17	IVS17+71A>G	
21	1444C>T	P482S
23	1634G>C	G545A
29	IVS28-5C>T	
33	3011T>C	L1004P
39	3594G>A	G1198G
	3684G>A	K1228K
42	4080A>G	P1360P
44	4207C>T	P1403S
47	4548A>G	V1516V
48	4932C>T	F1644F

membrane disease. This may be the case of HFB-4, which at the moment of the renal biopsy, looked like FBH, but in which the two affected members afterward developed significant proteinuria without renal failure. Although we did not find any *COL4A3/4* mutation in this family, we cannot exclude this possibility, because mutations in the *COL4A3/4* genes were found to be responsible for a large spectrum of phenotypes, as already pointed out by Heidet *et al.* (19).

Because of the difficulties inherent to linkage analyses, the present study on FBH is not only based on linkage analyses but also on mutation detection, which provides a direct and individual diagnosis. Up to now, only three mutations within the *COL4A4* gene had been reported in FBH families (20–22). However Heidet *et al.*, (19) recently reported a family with autosomal recessive AS and a mutation within the *COL4A3* gene. The parents of the affected sibling showed typical features of FBH, but they had not been biopsied. We analyzed all exons from the *COL4A4* and *COL4A3* genes and detected six new mutations. IVS23–1G>C should produce an aberrant splicing of exon 24 in the *COL4A4* gene. 3222insA and 31del11 are frameshift mutations. We detected three missense mutations (two in *COL4A3* and one in *COL4A4*) all of them affecting a glycine residue (G960R in *COL4A4* and G985V and G1015E in *COL4A3*). Missense mutations affecting glycines are frequent in the collagen genes. Glycine is the only amino acid small enough to fit the triple helix structure of the collagen network. However, changes affecting glycines have been considered as polymorphisms in the *COL4A3* (G43R) and *COL4A4* genes (G545A) (22,23). G43R is supposed to be a polymorphism, as this is the first glycine of the collagen domain. Glycine at position 545 (*COL4A4*) should not be critical to the overall stability of the helix. The three glycine-affecting mutations described here were not detected upon screening 200 control chromosomes; they are, therefore, likely to be the causative mutation in these families. Missense mutations affecting glycines should be tested in control population to consider them polymorphisms or mutations.

In this study we detected the pathogenic mutation in 60% of families (excluding one family with negative linkage to the *COL4A3/4* locus) and in 66.6% of families, if HFB-4, which seems to have something other than FBH, is excluded. This mutation detection is far lower than the rate of mutation detection for other noncollagenous genes using SSCA, reported by our group (36), but it is very similar to the one reported by Heidet *et al.* (19) for *COL4A3* using the same technique. It may be possible that some mutations are missed due to the large number of polymorphisms that make additional band shifts very difficult to detect. As a matter of fact, 81.2% of the DNA changes detected by SSCA in this study were polymorphisms (DNA changes not segregating with the disease or found in unaffected subjects). Additionally, large rearrangements or mutations in introns or regulatory elements of type IV collagen genes would not be detected by SSCA of *COL4A3/4* exons.

This study clearly demonstrates the main role of the *COL4A4* and *COL4A3* genes in the pathogenesis of FBH; however, it does not rule out the possibility that other genes expressed in the glomerular basement membrane may be involved in some families with FBH. Possible effects of *COLA43* and *COL4A4* mutations in FBH could be a reduced crosslinking in the type IV collagen network, a decreased content of type 4 collagen novel chains in the GBM and eventually a decreased thickness and stability of the GBM. FBH could be considered as an intermediate phenotype of AS in which a gene-dosage effect is present.

Molecular diagnosis of FBH is still not easily available, as (*1*) *COL4A3/4* genes are very large and difficult to screen for mutations, (*2*) no hot spots seem to exist, and (*3*) genetic heterogeneity is possible (24,25). The identification of other genes involved in FBH will be of particular interest for clarifying the pathogenesis of this disorder and perhaps for the development of an effective treatment for AS.

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