Benign Familial Hematuria due to Mutation of the Type IV Collagen α4 Gene

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Abstract

Benign familial hematuria (BFH) is characterized by autosomal dominant inheritance, thinning of the glomerular basement membrane (GBM) and normal renal function. It is frequent in patients with persistent microscopic hematuria, but cannot be clinically differentiated from the initial stages of Alport syndrome, a severe GBM disorder which progresses to renal failure. We present here linkage of benign familial hematuria with the COL4A3 and COL4A4 genes at 2q35-37 ($Z_{\text{max}} = 3.58$ at $G = 0.0$). Subsequently, a glycine to glutamic acid substitution was identified in the collagenous region of the COL4A4 gene. We conclude that type IV collagen defects cause both benign hematuria and Alport syndrome. Furthermore, our data suggest that BFH patients can be carriers of autosomal recessive Alport syndrome. (J. Clin. Invest. 1996; 98:1114–1118.) Key words: hematuria • Alport syndrome • basement membrane • collagen • mutation analysis

Introduction

Hematuria is the presenting symptom in a large number of renal disorders. If the hematuria occurs in childhood, is isolated and is of glomerular origin, the diagnosis is difficult to reach. Benign hematuria, Alport syndrome and IgA-nephropathy all present with persistent hematuria. Particularly, differentiation between Alport syndrome, which is a severe renal disorder, progressing to renal failure in most cases, and benign hematuria is mandatory for correct prediction of prognosis and genetic counseling. In benign hematuria electron microscopic analysis of renal biopsies shows thinning of the glomerular basement membrane (GBM) (1–3), while in Alport syndrome renal biopsies are characterized by irregular thickening and multilamination of the GBM (4). However, thinning of the GBM may be the only microscopic abnormality in younger Alport patients (5–7), which hampers the differentiation between the two disorders in childhood. Renal biopsies of 65 children with isolated hematuria persisting for at least one year revealed histological abnormalities consistent with Alport syndrome in eight cases and consistent with benign hematuria in 33 cases (3). A positive family history was detected in 23 of the 33 cases.

The major structural component of the GBM is formed by a type IV collagen network. As yet, six different type IV collagen chains have been identified and the respective genes were cloned. Mutations were detected in three type IV collagen genes in Alport syndrome. The majority was present in the X-linked type IV collagen α5 (COL4A5) gene, but recently mutations in the type IV collagen α3 and α4 (COL4A3/A4) genes have been reported in patients with the autosomal recessive form of this disease (8, 9). These latter two collagen genes are localized head to head on the tip of the long arm of chromosome 2 (10). Considering the similarities in GBM abnormalities, autosomal Alport syndrome and BFH could be the severe and mild forms of different molecular genetic defects in the same genes. Here we report linkage of the COL4A3/4 locus to BFH in a large Dutch family (11) and the identification of a pathogenic mutation in the COL4A4 gene.

Methods

Patients. The index patient (III:10; see Fig. 1) presented with hematuria at the age of 5 years. There were no concomitant abnormalities, i.e., glomerular filtration rate was normal and hypercalcuria was absent. There was also no hypertension. The morphology of the erthrocytes in the urine sediment indicated a glomerular origin of the cells. Audiography and fundoscopy revealed no abnormalities. Family history was negative for renal failure and deafness. One year later a renal biopsy was performed. Electron microscopy of the biopsy specimen showed regions with malformations of the glomerular basement membrane, typical for Alport syndrome, and regions which were thin. Further investigation of the family showed microscopic hematuria in both parents and his younger brother (III:11), but no other symptoms suggestive for Alport syndrome. Renal function was normal in the parents (II:7 and II:8; normal serum creatinine concentration). Microscopic hematuria was present in three sisters of the father (II:7) and in half of their off-spring, as well as in the grandfather (I:1; Fig. 1). Particularly important is that this 75-year-old man had a normal serum creatinine concentration. In the family of the mother (II:8) hematuria was also widely present. Her 76-year-old father had hematuria and a normal creatinine level, her mother was healthy. It was con-
cluded that the family history on both sides strongly supported the diagnosis BFH. The index patient might have inherited the disorder form both parents, which could explain the aberrant morphology of the biopsy specimen. He is presently 16 years old and symptoms are hematuria and recently developed proteinuria (0.5 grams/liter). Hematuria was defined as a condition with 10 or more erythrocytes per mm³ urine, established by the quantitative sediment method of Gade-holt (12). Individuals with borderline values were repeatedly analyzed. All family members could unambiguously be scored as either affected or unaffected.

**Linkage analysis.** Blood samples were collected from family members and genomic DNA was isolated by a salting out procedure (13). The COL4A3/COL4A4 CA repeat was PCR amplified using 125 ng of primers CA11F (5'-ATC TCT CAG GTG TCG TGC-3') and CA11R (5'-CTC ATT GAT ACA CAC AAA TGC A-3') and 50-100 ng DNA template in a standard Cetus buffer with 2 mM MgCl₂. After 3 min at 94°C, four initial cycles were performed of 1 min at 94°C, 1 min at 58°C and 1 min at 72°C followed by 30 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C. One of the primers was fluorescently labeled and the PCR product was analyzed on an ABI 373A Automated Sequencer using the Genscan software package (Applied Biosystems, Foster City, CA). A COL4A4 HaeIII RFLP was identified by SSCP and subsequent sequence analysis and is caused by a neutral mutation at the J198 glycine residue of the COL4A4 gene. PCR was performed on 100 ng genomic DNA using 100 ng of intronic oligonucleotides A41F9 (5'-AGGGACAAGA-3') (9) and 714 (5'-CTCATTGCTATTGGGAAGAG-GT-3'), 200 μM dNTPs, 5% DMSO in Ampli-Taq buffer containing 67 mM Tris-HCl (pH = 8.8), 16.6 mM (NH₄)SO₄, 6.7 mM MgCl₂, 0.17 mg/ml BSA (Boehringer Mannheim, Germany) to a total volume of 25 μl. The sequence of oligo 714 is the reverse of oligonucleotide A41F9 (9). After 5 min of denaturation at 96°C 1 U Ampli-Taq DNA polymerase (Perkin-Elmer, Norwalk, CT) was added and 30 cycles of PCR were started (denaturation at 94°C for 1 min, annealing at 62°C for 1 min and extension at 72°C for 1 min and 30 s). If the HaeIII site is present, digestion of the PCR fragment (260 bp) gives two bands of 182 and 78 bp (data not shown).

Two point and multi-point lod scores were calculated using the MLINK and LINKMAP options of the LINKAGE package computer program (version 5.03) (14). BFH was defined as an autosomal dominant disorder with complete penetrance. The disease frequency was estimated to be 0.0001.

**Mutation analysis.** RNA was isolated from peripheral blood lymphocytes (PBL) or EBV transformed PBL from affected and normal individuals by RNAzol extraction (15). COL4A3 and COL4A4 cDNA fragments were generated by RT-PCR analysis as described before (8, 16). Oligonucleotides were designed to amplify COL4A3 and COL4A4 cDNA by multiple overlapping cDNA fragments. DNA fragments were sequenced on an ABI 373A automated sequencer. Reaction conditions using dideoxy™-terminators were according to a protocol provided by the manufacturer (Applied Biosystems, Foster City, CA). The Gly897Glu mutation was identified in the PCR fragment, flanked by outer primers 945 (5'-GCCGAAG-GGTGACATTGTTGATCA-3') starting at position 1871 and 946 (5'-CCCTTCAGAAGTGCAACTCCAG-3') starting at position 4074 (17) in a first round of 35 cycles; denaturation for 1 min at 92°C, 1 min 30 s of annealing at 60°C and extension for 3 min at 72°C. Two microelutes out of the first PCR reaction was used as template for a second round of 35 cycles using inner primers F100 (5'-AAGGCGC-TCGCCAGACTCCCA-3') at position 2750 and R101 (5'-CGGGAG-GTCTATTGGGCTC-3') at position 3017 (17); denaturation for 1 min at 92°C, 1 min 30 s of annealing at 55°C and extension for 2 min at 72°C. The Gly897Glu mutation created an Alul restriction site and was screened at the genomic DNA level by PCR amplification using oligonucleotides F100 (5'-AAGGCGCTCCCCGGACTCCTCCCA-3') at position 2750 and R101 (5'-CGGGAG-GTCTATTGGGCTC-3') at position 3017 (17); denaturation for 1 min at 92°C, 1 min 30 s of annealing at 55°C and extension for 2 min at 72°C. The Gly897Glu mutation created an Alul restriction site and was screened at the genomic DNA level by PCR amplification using oligonucleotides F100 (5'-AAGGCGCTCCCCGGACTCCTCCCA-3') and 1106 (5'-ACCTGGAGAACCAGGTAG-3') digestion with Alul restriction enzyme (GIBCO BRL, Gaithersburg, MD). If the mutation is present the 104-bp PCR fragment is cleaved in two fragments of 86 and 18 bp. The smallest fragment is not visible.

**Results**

**Linkage analysis.** Linkage analysis was performed with two novel polymorphic markers from the COL4A3/COL4A4 locus. The most informative marker was a CA repeat, isolated from a YAC clone with an insert of 540 kb, containing both COL4A3 and COL4A4 genes (18). The other marker was a

![Figure 1](image-url)
HaeIII RFLP in one of the exons of the COL4A4 gene. Because the genomic structure of COL4A4 is not clarified yet, the exact numbering of exons is unknown, but most likely exon 8 is involved (counting from the 3' end). The RFLP is caused by a polymorphism, a neutral G to A nucleotide substitution at Glycine198 (GGG into GGA) (17). The allele frequencies of this polymorphism were 0.5/0.5 (70 unrelated individuals tested).

**Figure 2.** Gly897Glu substitution in the type IV collagen α4 chain in BFH. Sequence analysis of type IV collagen α4 cDNA of the index patient and a normal control. The mutation, which changes the glycine (G) codon 897 GGG into the glutamic acid (E) codon GAG is indicated by an arrow. Type IV collagen chains consist of collagenous and non-collagenous (NC) domains. Three type IV collagen chains form a triple helix, which is interrupted by the substitution of a conserved glycine residue.

**Figure 3.** Segregation of Gly897Glu mutation in the BFH family. The presence of the mutation creates an additional Alu I restriction site. Cleavage of the 104 bp PCR fragment in two shorter fragments of 86 and 18 basepairs is indicative for the presence of the mutation. Only the affected family members (filled symbols) show the shorter DNA fragment (86 bp), the smallest fragment of 18 bp is not visible. DNA fragment lengths are indicated in basepairs (bp).
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Discussion

For several reasons, the Gly897Glu mutation in the type IV collagen α4 chain is likely to be the pathogenic mutation in the BFH family. First, the mutation changes a glycine in a conserved collagenous triple helical domain in the type IV collagen α4 chain (17). Every third amino acid is a glycine, because it is the only residue small enough to fit into the center of the triple helical molecule (19). In glycine residues are substituted by bulkier amino acids such as glutamic acid the triple helix structure will be distorted (Fig. 2). Unlike fibrilar collagens, type IV collagen triple helices contain 21-26 interruptions, which are assumed to provide flexibility to the molecule. However, the positions of these interruptions are highly conserved during evolution and the generation of an additional interruption may interfere with correct folding of the intrinsic type IV collagen trimers, consisting of type IV collagen α3, α4, and α5 chains. Substitutions for glycine in the collagenous triple helical domain are also a common cause of other collagen disorders, like osteogenesis imperfecta and Ehlers-Danlos syndrome (20). Furthermore, the mutation cosegregates with BFH in our family and, finally, the mutation is absent from the unaffected family members and 50 unrelated controls (data not shown).

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References


