COL4A4 mutation in thin basement membrane disease previously described in Alport syndrome

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Background. Carriers of autosomal-recessive and X-linked Alport syndrome often have a thinned glomerular basement membrane (GBM) and have mutations in the COL4A3/COL4A4 and COL4A5 genes respectively. Recently, we have shown that many individuals with thin basement membrane disease (TBMD) are also from families where hematuria segregates with the COL4A3/COL4A4 locus. This study describes the first COL4A4 mutation in an individual with biopsy-proven TBMD who did not have a family member with autosomal-recessive or X-linked Alport syndrome, inherited renal failure, or deafness.

Methods. The index case and all available family members were examined for dysmorphic hematuria >50,000/mL using phase contrast microscopy and for segregation of hematuria with the COL4A3/COL4A4 and COL4A5 loci using DNA satellite markers. COL4A4 exons from the index case were then studied using the enzyme mismatch cleavage method, and exons that demonstrated abnormal cleavage products were sequenced.

Results. Hematuria in this family segregated with a haplotype at the COL4A3/COL4A4 locus (P = 0.031) but not with haplotypes at the COL4A5 locus. A mutation in COL4A4 that changed C to T resulting in an arginine residue being replaced by a stop codon (R1377X) was demonstrated in exon 44, which encodes part of the α4(IV) collagen sequence close to the junction with the noncollagenous domain. This mutation was present in all five family members with hematuria, but not in the four unaffected family members, 33 unrelated individuals with TBMD, or 22 nonhematuric normals.

Conclusions. R1377X has been described previously in a compound heterozygous form of autosomal-recessive Alport syndrome [1] and in autosomal-recessive Alport syndrome [1]. Although carriers of X-linked Alport syndrome often also have thin membranes [2], they can usually be identified by localized regions of GBM lamellation.

Key words: collagen type IV, hematuria, glomerular basement membrane, inherited disease, gene mutation. Here we describe a family with TBMD caused by a mutation in the COL4A4 gene. This is the first time that a mutation has been described in TBMD where there was no family member with autosomal-recessive (or X-linked) Alport syndrome. Furthermore, this mutation
had been demonstrated previously in a compound heterozygous form of autosomal-recessive disease [8]. Thus, this mutation that causes TBMD in the absence of a family history of autosomal-recessive Alport syndrome can still represent a carrier state for autosomal-recessive Alport disease.

**METHODS**

**Patients**

The index case was first investigated 18 years previously when he was 59 years old. At that time, he had 75,000 dysmorphic red blood cells (RBC)/mL on phase contrast microscopy of a midstream urine specimen, approximately 500 mg proteinuria per day, and a normal serum creatinine [13]. His 44-year-old daughter and her three children aged 13, 15 and 18 currently all had >50,000 dysmorphic RBC/mL in their urine.

The index case’s renal biopsy at presentation contained eight glomeruli, most with mild-to-moderate mesangial thickening and occasional areas of segmental sclerosis, and with one completely sclerosed glomerulus. Only minor patchy interstitial fibrosis and tubular atrophy, occasional chronic inflammatory and foam cells, and mild vascular changes were found. Mesangial and paramesangial IgM was present. On electron microscopy, there was widespread attenuation of the GBM, which averaged 196 nm (112 to 280 nm), some irregular flouccular expansion of the lamina rara interna, occasional intramembranous spherical electron dense bodies, and generalized foot process effacement. There was no lamellation of the GBM. The diagnosis of TBMD with secondary focal glomerulosclerosis was made [14].

None of the family members who were examined had renal impairment, a clinically detectable hearing loss, anterior lenticus, or retinopathy typical of X-linked or autosomal-recessive Alport syndrome. Furthermore, there was no family history of renal failure or inherited deafness.

**Linkage studies**

DNA from peripheral blood leukocytes or buccal smears was extracted and amplified using standard techniques and published conditions. Linkage to the COL4A5 locus was examined using the 2B6 and DXS456 markers [15, 16] and to the COL4A3/COL4A4 locus using the CA11, D2S351, and D2S401 markers and a COL4A4 HaeIII intragenic restriction fragment length polymorphism (RFLP) [7, 17]. Each 10 μL reaction mix contained 50 ng genomic DNA, 100 ng primers with the 3’ primer end labeled with γ32P-ATP [8], which for wild-type DNA had been end-labeled with γ32P-ATP using T4 polynucleotide kinase (Geneworks, Adelaide, South Australia, Australia), 1 mmol/L dNTP (Pharmacia, Uppsala, Sweden), 1 μL of 10 × reaction buffer, 2.5 mmol/L MgCl₂, and 0.5 μ Taq polymerase (Geneworks). After amplification, 5 μL of formamide stop solution [95% formamide, 20 mmol/L ethylenediaminetetraacetic acid (EDTA)] was added to each tube, which was incubated at 95°C for 30 minutes and then chilled. Six microliters from each reaction mix was then electrophoresed in a 7% denaturing polyacrylamide gel at 50°C for two to four hours. Alleles were determined by exposing the gel to x-ray film (Biomax; Kodak, Rochester, NY, USA) and assigned numbers according to band size. Genotypes for the COL4A4 RFLP were determined after amplification, restriction with HaeIII (Boehringer Mannheim, Mannheim, Germany) at 37°C for four hours and electrophoresis in a 1% agarose gel.

Haplotypes were constructed at the COL4A5 and COL4A3/COL4A4 loci for affected and unaffected family members. The family was too small for formal linkage studies, but the probabilities of hematuria segregating with haplotypes at each locus were calculated [11].

**Mutation detection**

The enzyme mismatch cleavage method was used to detect the mutation in the index case [18]. With this method, patient and radiolabeled wild-type DNA form heteroduplexes that are digested by bacterial endonucleases wherever there are mismatches and mutations are demonstrated by novel banding patterns on autoradiography. This technique demonstrates at least 95% of all mutations.

Patient and wild-type DNA were amplified separately using specific primers for each exon of COL4A4 [8], which for wild-type DNA had been end-labeled with γ32P-ATP. The amplification products were purified using “Freeze ‘N Squeeze” DNA gel extraction spin columns (Quantum Prep; Bio-Rad, Hercules, CA, USA). Fifty nanograms of each patient and wild-type DNA were then heated to 95°C for 6.5 minutes, 65°C for 30 minutes, and 25°C for 20 minutes to allow heteroduplex formation. Five microliters of a 10 × restriction buffer [0.5 mol/L Tris, 100 mmol/L MgCl₂ 5 mmol/L dithiothreitol (DTT), bovine serum albumin (BSA) 1 mg/mL; Boehringer Mannheim] were added, and the reaction mix was incubated with 250 units of T7 endonuclease I in a total volume of 50 μL at 37°C for 20 to 25 minutes, precipitated on ice with 100% ethanol; the precipitate resuspended in 5 μL of stop solution (95% formamide, 20 mmol/L EDTA). This was electrophoresed in a 7% acrylamide gel containing 5.6 mol/L urea and 32% formamide at 80 watts for approximately one hour per 100 bp of product. The gel was exposed to x-ray film at ~80°C overnight.

Exons containing possible mutations were then amplified (BigDye Terminator Cycle Sequencing Ready reaction), purified, and sequenced in an automated DNA sequencer. The exon containing the mutation was amplified in the other eight family members, 33 unrelated

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individuals with TBMD, and 22 normal individuals who did not have hematuria.

This project had the approval of the Human Research Ethics Committee of the Austin and Repatriation Medical Centre, and all subjects provided signed informed consent.

**RESULTS**

In this family, hematuria segregated with the 2121 haplotype at the COL4A3/COL4A4 locus \((P = 0.031)\) and not with the COL4A5 locus (Fig. 1).

A C to T substitution at nucleotide 4337 resulting in an arginine being replaced by a stop codon \((R1377X)\) [9] was demonstrated in exon 44 of COL4A4 (Fig. 2). This encodes part of the collagen sequence close to the junction with the noncollagenous domain of the \(\alpha4(IV)\) molecule. There were no mutations in any other COL4A4 exons in the index case from this family.

R1377X was present in all five members of the family with hematuria but not in the four unaffected family members, 33 unrelated individuals with TBMD, or 22 nonhematuric normals.

**DISCUSSION**

To our knowledge, this is the first example of a mutation being demonstrated in an individual with biopsy-proven TBMD where there were no family members known to have autosomal-recessive Alport syndrome and
where TBMD did not represent an obvious carrier state for this condition. However, the previous demonstration of an identical mutation in autosomal-recessive Alport syndrome indicates that this mutation can result in autosomal-recessive disease in the compound heterozygous form as well as cause TBMD. Thus, individuals with TBMD without a family history of autosomal-recessive Alport syndrome can still be carriers of this condition.

The presence of dysmorphic or “glomerular” hematuria was used to identify affected individuals in this family because it is the most common clinical feature in TBMD and persists throughout life [19]. All hematuria in family members contained at least three different populations of RBCs, indicating that it was glomerular rather than nonglomerular in origin [13].

Hematuria in the family described here segregated with the COL4A3/COL4A4 locus, and each affected family member had the C to T nucleotide substitution that resulted in a nonsense mutation and truncation of the corresponding α4(IV) collagen chain. At the most, one third of mutations in autosomal-recessive and X-linked Alport syndrome cause stop codons, and these are always pathogenic [20]. In this family, all individuals with the mutation had hematuria, whereas the penetrance of hematuria is usually only 80% at the COL4A3/COL4A4 locus (abstract; Dagher et al., J Am Soc Nephrol 10:432A, 1999). Whether the penetrance of hematuria in this family was increased because the underlying mutation resulted in a stop codon is not clear.

The mutation described in this study is identical to one demonstrated previously in a family with autosomal-recessive Alport syndrome [8]. Most families with Alport syndrome have different mutations, with more than 300 described to date in X-linked inheritance and approximately 30 in autosomal-recessive disease. However, there is a report of a shared or common mutation in nine families with X-linked Alport syndrome, and the demonstration of a shared haplotype at the COL4A5 locus and careful genealogical studies have indicated that this was due to a founder effect [21]. Our family was of British origin, but we do not know the nationality of the previously described family nor their haplotype at the COL4A3/COL4A4 locus [8]. We did not find this mutation in any of 33 further unrelated individuals with TBMD who were tested.

Finally, it remains unclear whether most individuals with TBMD simply have mutations in the same genes that are affected in autosomal-recessive Alport syndrome or whether the majority are carriers of autosomal-recessive Alport syndrome and compound heterozygotes and homozygotes will always develop the characteristic clinical phenotype of renal failure, deafness, and ocular abnormalities. A comparison of a larger series of mutations in autosomal-recessive Alport syndrome and TBMD should indicate which explanation is more likely.

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