Novel COL4A4 splice defect and in-frame deletion in a large consanguine family as a genetic link between benign familial haematuria and autosomal Alport syndrome

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Abstract

Background. Alport syndrome (AS) is a common hereditary cause for end-stage renal failure due to a defect in type IV collagen genes. The molecular pathogenesis of benign familial haematuria (BFH) is not fully understood. Evidence from linkage analyses and mutation studies point to a role of the COL4A3/COL4A4 genes. The present study describes molecular changes of the COL4A4 gene that cause both diseases: autosomal recessive AS and BFH in a consanguine family with a 400-year-old history of haematuria.

Methods. RNA and DNA were isolated and analysed by RT–PCR, PCR, DNA and cDNA sequencing, and Southern blotting. Evaluation of family members comprised creatinine clearance, urine analysis, audiometry and past medical history.

Results. Forefathers of this family moved to a German village in the 17th century. Sporadic episodes of macrohaematuria have been reported ever since. Numerous family members with haematuria including the parents of the index family were heterozygous for a splice defect eliminating exon 25 from the α4(IV) cDNA. The daughter (15 years old, creatinine clearance 27 ml/min, proteinuria 5 g/day, hearing loss) was homozygous for the mutation, while the son (22 years old, creatinine clearance 68 ml/min, proteinuria 11 g/day, hearing loss, splitted and thickened glomerular basement membrane) was heterozygous. Further analysis showed a second mutation, an 18 bp in-frame deletion in exon 25, for which numerous family members were heterozygous, and both children were homozygous.

Conclusions. The COL4A4 splice defect causes BFH-phenotype in heterozygous, and AS in homozygous state. The clinical spectrum of heterozygous individuals reaches from macrohaematuria, intermittent microhaematuria to isolated deafness. The 18 bp in-frame deletion aggravates the phenotype in the compound heterozygous son. These results give further evidence that BFH and autosomal AS are in fact both type IV collagen diseases.

Keywords: deafness; extracellular matrix; genetic disorder; kidney failure chronic; thin glomerular basement membrane disease; type IV collagen

Introduction

Alport syndrome (AS) is a hereditary nephropathy characterized by a family history of haematuria and proteinuria, progressive renal failure, sensori-neural deafness and typical ocular changes [1,2]. The disease is caused by mutations in type IV collagen genes. Type IV collagen is a major constituent of basement membranes. While the α1(IV) chains are found ubiquitous in basement membranes, the α3, α4 and α5(IV) chains show a restricted distribution and are specifically expressed in the glomerulus, inner ear and eye [3]. There is evidence that mutations of one COL4A3, 4 or 5 gene not only alter or abolish expression of the corresponding α(IV) chain, but also lead to an abnormal basement membrane with decreased or absent α3, α4 and α5(IV). How this initiates the progressive nephritis and scarring observed in AS is not well understood [3–6]. The first sign of this phenotypically heterogeneous disease is haematuria, progressing to end-stage renal failure. In electron microscopy Alport patients typically show an altered structure of the glomerular basement membrane in form of irregular thickening and splitting, uniform thinning may be observed in younger patients [7].

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The mode of inheritance of AS is X-chromosomal in > 80% [2]. In 10–15% of the families the genealogical tree is compatible with an autosomal trait. Two type IV collagen genes, COL4A3 and COL4A4, both localized on chromosome 2 turned out to be mutated in autosomal recessive AS. The frequency of the COL4A5 gene is estimated at 1:5000 [2,5], the frequency of autosomal recessive AS at 1:50 000.

The gene-defect causing benign familial haematuria (BFH or thin basement membrane disease) is not known in most cases. The autosomal-dominant disease is characterized by haematuria without deterioration of renal function and by thinning of the glomerular basement membrane [8]. Because of these facts BFH is hard to distinguish from early autosomal AS. Five per cent of all kidney biopsies are diagnosed being ‘thin basement membrane disease’ by electron microscopy, this diagnosis is made in ~ 20% of patients with asymptomatic haematuria. When taking urine samples of family members of these patients family history is positive for haematuria in 92%, 47 ± 6% of family members are affected (hint to autosomal inheritance) [9].

Linkage analysis in a Dutch family with BFH identified a pathogenic mutation in the COL4A4 gene [10]. Other studies describe mutations in the COL4A4 gene associated with BFH, mutations that cause autosomal recessive AS, and linkage of BFH to the COL4A3/COL4A4 (or COL4A5) locus in ~ 50% of cases with thin basement membrane disease [11–16].

Here we describe a large, consanguine family with a 400-year-old history of haematuria caused by a COL4A4 splice mutation and an 18 bp in-frame deletion in exon 25 of the COL4A4 gene. The spectrum of phenotype in heterozygous carriers reaches from macrohaematuria and intermittent microhaematuria to isolated deafness. In homozygous form, these mutations cause autosomal AS. Our results give further evidence, that COL4A4 mutations can cause both, BFH and autosomal AS, and can lead to a broad spectrum of clinical phenotypes in heterozygous carriers.

**Subjects and methods**

**Clinical evaluation**

Past medical history was taken from all living family members including haematuria, proteinuria, hearing loss, eye symptoms and hypertension. Patients were screened by blood test (serum creatinine, urea, potassium and serum albumin), urine analyses (sediment analysis, polyacrylamide-gel-electrophoresis of proteins) and for lenticonus and sensorineural deafness. Church records starting in the early 17th century gave additional information about past family history.

**Southern blot analyses**

Genomic DNA was isolated from blood and preparation for Southern blotting was performed as described earlier [17–19]. Genomic DNA was digested overnight with *PstI*, precipitated and loaded on a 0.7% TBE agarose gel, followed by capillary blotting on a nylon filter. Filters were hybridized overnight with 10 ng DIG-dUTP labelled COL4A4 cDNA specific probe (Boehringer Mannheim) per ml Easy-Hyp hybridization solution. Washing and chemiluminescence detection were performed according to the manufacturers protocol (Boehringer Mannheim).

**Reverse transcription PCR and direct sequencing**

Lymphocytes were isolated from EDTA blood. Total RNA was isolated (InViSorb RNA Kit, InViTek). Transcription into cDNA was performed with Superscript reverse transcriptase according to the manufacturers protocol (Gibco BRL), using both Poly-T and random primers. 2 μl out of 200 were used for amplification of COL4A4 cDNA fragments, using sequence specific nested primers. One millilitre from the first PCR reaction was used as a template in the second round. Standard PCR was performed with the following conditions: 5 min at 95°C, followed by 35 cycles (first round) or 25 cycles (second round) of 30 s at 95°C, 30 s at 55°C, 90 s at 72°C and a final elongation step of 5 min at 72°C.

Purified PCR products were sequenced with the PRISM dye terminator cycle sequencing kit (Perkin Elmer), using 50 ng template and the same primers as for PCR amplification. Sequencing analysis was subsequently performed on an ABI sequencer 373.

**PCR**

Forward-primer 24A (ACTTTACCCTCTGCTGATAAA) and 25A (TTTCTGACCCCTTAAGCCCAT) fitted into exon regions of exon 24 and 25. Primers 25B (TAAACACTTGACCCCAAG), 25B2 (CCTCACAACCTGGTGCCCTGC) and 25Bdel18 (CCTGGGTTGCCCTGGAACCCTCTG) served as reverse primers. PCR amplification was performed in a total volume of 50 μl, using 100 ng DNA, 20 pmol of each primer, 2 mM MgCl₂, 0.2 mM dNTP and 1 U Taq polymerase (Promega) for 35 cycles. Annealing temperatures varied between 50 and 58°C for 30 s, elongation times varied between 30 and 150 s.

**Results**

**Family history**

We followed the index family (C5, C6, D5, D6 and D7) for the last 12 years (Figure 1). The son D6 was diagnosed having AS by his clinical symptoms and by kidney biopsy in 1990. Kidney biopsy showed multilamellar splitting with thick and thin glomerular basement membrane. D6 today is 22 years of age, currently leading to nephrotic syndrome with proteinuria and macrovisceral splitting with thick and thin glomerular basement membrane. D6 today is 22 years of age, currently leading to nephrotic syndrome with proteinuria of 8–11 g/day. At present, his creatinine clearance is 68 ml/min, declining by ~ 5 ml/min (7.5–10%) per year (Figure 2). Eye-examinations for macula spots and lenticous were negative for all family members. The girl D5 was found to have persistent microhaematuria and proteinuria at the age of six. She has been wearing a hearing aid since she was 8. Today, at the age of 15, she has nephrotic syndrome with
proteinuria of 5 g/day. Her creatinine clearance is 27 ml/min and declines by 8–10 ml/min (20–25%) per year (Figure 2). Both children D5 and D6 developed hypertension. Her other brother D7, who is homozygous for the unaffected allele, appears healthy and has no symptoms such as haematuria.

Fig. 1. Forefathers of the index family moved to a small village in Saarland (Western part of Germany) in ~1620. Ten sons (girls were not mentioned in church records at that time) and following generations stayed in same area, that consisted of a very few, isolated villages at that time. Family members were known to have sporadic episodes of macrohaematuria for many generations ever since they lived in this area. Therefore, it is likely that the forefathers already carried the ‘founder-mutation’.

The maternal side of the index family reported intermittent microhaematuria and rare episodes of macrohaematuria and chronic (not end-stage) renal disease in patient A3. Interestingly, patients B6 and B7 had sensori-neural deafness since their first decade of life, which was not caused by recurrent ear-infection. B7 was found to be a heterozygous carrier of the mutation and had hypertension; however, haematuria could not be found in either patient B7 nor in patient B6 in two separate urine samples. The paternal side reported persistent micro- and recurrent macrohaematuria. Most of the affected family members above the age of 45 had hypertension. Patient B2 had ‘weak kidneys’, but did not suffer from end-stage renal failure.

Consanguinity was negated, however, both families originate from the same village. The total population in that area was 18 700 people (1990). When visiting the family at their home for follow up studies, we had some problems to find the right address, as several families with the same last name lived in the same street. Sixty-one people of the village carry the same last name than our index family (1:300), compared with 1:100,000 in the control group (population of Cologne, Frankfurt, Munich and Berlin). As the
mother’s birth name is different, it can be assumed, that a lot more people are consanguineous in this area and might carry the COL4A4 mutation.

**RT–PCR of the COL4A4 gene and direct sequencing of cDNA (Figure 3)**

Nested PCR with primers 25A and 25B of the index family resulted in a shorter fragment (compared with controls). Son D6 and both parents C5 and C6 were heterozygous for this mutation, the daughter D5 is homozygous (Figure 3A). Control RT–PCR with primers 25A and 25B2 (Figure 3B) showed one unaffected allele in both parents, no unaffected allele in the daughter and an additional, shorter fragment in the son. Subsequent direct sequencing of the cDNA–PCR product and comparison with the published sequence of the COL4A4 cDNA revealed a deletion of 184 bp at position 2014 and an additional 18 bp deletion in the son D6. The 184 bp cDNA deletion was shown to be exon 25 of the COL4A4 gene (Figure 3C), the 18 bp deletion was shown to be an in-frame deletion within exon 25 of the COL4A4 gene.

To verify our data from RNA analysis on the genomic level, we performed Southern blotting of the siblings, their parents and two controls (data not shown). Genomic DNA was digested with PstI. All family members show an abnormal restriction pattern, both parents having the same abnormal restriction pattern.
We followed the index family since 1990, when the manifestations of AS, supported by the observation of severe symptoms in females (D5) and transmission from father to son (C2, C3, C4, C5, D1, D2, D4, D6). Back in 1990, family history was unclear, if an autosomal recessive or autosomal-dominant trait of AS was found. Meanwhile, the severe symptoms of the homozygous daughter D5 and much milder symptoms of all heterozygous family members make an autosomal recessive form of AS very likely.

A long family history of haematuria without end-stage renal failure led us to the hypothesis, that the COL4A4 mutation causes BFH in heterozygous state. Most family members show intermittent macrohaematuria and persistent microhaematuria, some only show intermittent microhaematuria. Can these symptoms be diagnosed as BFH? With the molecular-genetic tools in our hands to diagnose heterozygous state in all family members, there is no reason to perform kidney biopsies to support our hypothesis. However, there are several other facts supporting our hypothesis.

More than 50% of male and female family members have haematuria. The genealogical tree is compatible with an autosomal-dominant trait for haematuria, as typical for BFH [8–10,18]. More than 90% of affected heterozygous family members have a normal renal function. Two members had chronic renal failure (reported as ‘bad kidneys’) above the age of 60, but did not die from renal failure or required dialysis. Having a history of intermittent macrohaematuria for almost 400 years, the haematuria in our index family can indeed be called familial and benign.

On the paternal side, the COL4A4 mutation causes intermittent macrohaematuria in nearly all affected family members for nearly 400 years. Most of the affected family members > 45 years of age have (mild) hypertension and were placed on ACE-inhibitors because of their nephroprotective and antifibrotic properties in type IV collagen diseases [20]. Hypertension might be caused by the underlying renal disease. In contrast, on the maternal side the same mutation leads to a different phenotype: intermittent microhaematuria and in some cases hearing loss. None of the healthy unaffected family members suffers from renal hypertension, haematuria or hearing loss. All paternal and maternal symptoms can as well be found in heterozygous carriers of COL4A5 mutations. However, it seems unusual, that the same mutation causes isolated hearing loss without haematuria in one family member and macrohaematuria without hearing loss in the other. This might be explained by a somewhat different genetic background of the paternal family members compared with the maternal family members. Another explanation could be an incomplete penetrance of the haematuria, and the hearing loss having another cause. The birth names on the maternal side are different to the father’s last name for at least four generations. Even though the father’s last name is very common in that area, consanguinity was negated and—according to church records—seems to be unlikely for at least the last four generations. Therefore, intermittent macrohaematuria on one hand and hearing loss or intermittent microhaematuria on the other seem to be a spectrum of clinical phenotypes caused by the same mutation, that are influenced by a different genetic background.

The splice site mutation eliminates exon 25 of the COL4A4 gene, producing a shift in the reading frame that leads to a truncated protein missing half of the
collagenous domain and the complete NC1 domain. As one would expect from similar mutations found in the COL4A5 gene [19], the homozygous COL4A4 mutation (daughter D5) leads to a severe phenotype with end-stage renal failure before the age of 18. In compound heterozygous form (son D6), the 18 bp in-frame deletion within exon 25 aggravates the 'benign' phenotype of carriers such as patient C5 to a 'mild' Alport-phenotype with later onset of renal failure.

The frequency of autosomal recessive AS is estimated at 1:50 000 [2]. Knowing this frequency, the Hardy–Weinberg law calculates the frequency of homozygous carriers for autosomal recessive AS to be heterozygous carriers for autosomal recessive AS. The data are in accordance to previous studies, finding 1% of the population is estimated at 1:50 000 [2]. Therefore, BFH is a common disease with end-stage renal failure before the age of 18. The frequency of autosomal recessive AS is estimated to be heterozygous carriers for autosomal recessive AS. The data are in accordance to previous studies, finding 1% of the population is estimated at 1:50 000 [2]. Therefore, BFH is a common disease that is frequently diagnosed by kidney biopsy. In contrast, other studies did not find linkage to the COL4A3/4 locus in some families with BFH [21]. Having identified this new pathologic mutation in our family and numerous family members further evidence was found for the possible close relation in etiology of autosomal recessive AS and BFH.

Conflict of interest statement. None declared.

References


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