Mutations in the type IV collagen α3 (COL4A3) gene in autosomal recessive Alport syndrome


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A group of 22 unrelated patients with sporadic or non-X-linked Alport syndrome were screened for mutations in the non-collagenous domain of the type IV collagen α3 (COL4A3) chain gene. The five 3'-exons of this gene, located on chromosome 2qter, were tested by single strand conformation polymorphism analysis and direct sequencing. One patient was heterozygous and another homozygous (Mochizuki et al., Nature Genetics, in press) for a deletion of five nucleotides. A third patient appeared to be a compound heterozygote for two different nonsense mutations. In two patients and the father of a deceased patient we found a heterozygous substitution of an evolutionary conserved leucine by proline. However, segregation data of the mutation and a COL4A3/COL4A4 CA-repeat marker in their families argued against a causative role of the missense mutation. Even drastic changes of strongly conserved amino acids, as in the Leu36Pro case, may not be significant. Autosomal recessive inheritance due to pathogenic COL4A3 mutations accounts for at least 13% of Alport syndrome cases in this sample. It is concluded that COL4A3 is a major gene in the genetically and clinically heterogeneous Alport syndrome.

INTRODUCTION

Alport syndrome is a progressive hereditary renal disorder with characteristic ultrastructural lesions of the glomerular basement membrane (GBM) (1). Patients have been classified by the severity and nature of the clinical features, combined with genetic segregation data in Alport families (2). In the vast majority of these families, Alport syndrome segregated as an X-linked dominant trait (3–5). More than 60 different mutations have now been detected in Alport patients in the X-chromosomal COL4A5 gene (Second International Workshop on Alport syndrome, February 1993, New Haven) (6). A second X-chromosomal type IV collagen chain gene (COL4A6) has been identified head to head to COL4A5 (7). Deletions including the 5' ends of both the COL4A6 and COL4A5 genes have been described in four cases of Alport syndrome in combination with oesophageal leiomyomatosis (7).

Apart from X-linked pedigrees, a smaller number of families has been described for which autosomal recessive inheritance was suggested (8,9). Three independent lines of evidence pointed to a crucial role of the COL4A3 gene product in a subset of Alport patients: (i) immunohistochemical studies have revealed the absence of reaction of Goodpasture antisera with the GBM of some Alport patients (autoantibodies against the non-collagenous (NC) domain of COL4A3) (10,11); (ii) anti-renal allograft antibodies from patients with an anti-GBM nephritis after kidney transplantation were shown to be directed against the COL4A3 NC domain (12); and (iii) in autosomal recessive Alport syndrome genetic linkage was demonstrated with the COL4A3/COL4A4 locus at the terminal region of the long arm of chromosome 2 (13). Recently, we have described the first mutations in the COL4A3 gene in female patients from consanguineous marriages (14). In the last few years we have collected 50 unrelated Alport patients with and without family history. This sample consists of 18 sporadic (15 males and three females), 28 X-linked, one autosomal dominant and three autosomal recessive cases. Although male patients predominated in the sporadic group, making the involvement of an X-linked gene for the majority likely, we decided to screen the entire group of sporadic and non-X-linked cases for mutations in the NC domain of COL4A3. This enabled us to evaluate the prevalence of mutations in the COL4A3 gene in Alport syndrome.

RESULTS

Human genomic DNAs from 21 unrelated Alport patients and the parents of one deceased patient (no. 6537) were screened by single strand conformation polymorphism (SSCP) analysis for mutations in the five exons encoding the NC domain of the COL4A3 gene. For exons 1, 2 and 3, counting from the 3' end of the gene, no mobility shifts were observed (data not shown). For exon 4 one of the patients (no. 5178) showed a single-stranded fragment of increased mobility, in addition to the normal fragment (data not shown). Screening of exon 5 revealed aberrant fragments in four patients, including the patient with the exon 4 abnormality. In one of these cases (no. 6447) the mobility of the exon 5 double-stranded band was increased and heteroduplexes between normal and mutant strands were identified (Fig. 1, lane 11). This suggested a small deletion in exon 5. Surprisingly, the DNA of patient 5178 showed a mobility shift of the double-stranded band, which might reflect a
heteroduplex, and only barely visible shifts of the single stranded bands (Fig. 1, lane 1). An identical single-stranded fragment shift was further identified among two unrelated patients [no. 4014, no. 5639 (Fig. 1, lane 7)] and the father of deceased patient 6537.

Apart from the aberrant band, all individuals showed also the normal band.

Direct sequence analysis was performed to determine the nature of the variants. Patient 6447 with the shorter double-stranded exon 5 fragment was heterozygous for a deletion of five nucleotides (CTTTT) in exon 5 (data not shown). This sequence was duplicated in normal individuals (CTTTTCTTTT), but was present only once in the patient. The predicted result of this frameshift mutation is a truncated protein with a non-functional NC domain (Fig. 2). An affected brother and sister from another family, who were homozygous for this mutation, have been reported elsewhere (14). Patient 5178 with an altered SSCP pattern in both exon 5 and 4 showed an Arg43Stop mutation (CGA—TGA) in exon 5 and a Ser86Stop (TCA—TGA) in exon 4 (counting from the first amino-terminal amino acid of the NC domain). To confirm that both COL4A3 genes were affected, we sequenced individual COL4A3 cDNA clones from this patient. COL4A3 cDNA was generated from peripheral blood lymphocytes by reverse transcription—polymerase chain reaction (RT—PCR) and cloned in vector pCR-Script™SK. Sequence analysis of seven clones revealed in three clones the Arg43Stop mutation and in four clones the Ser86Stop (data not shown). This result proved that the patient indeed was a compound heterozygote for the different nonsense mutations. It was predicted that the NC domains of the COL4A3 proteins of this patient contained only 42 and 85 amino acids, respectively (Fig. 2).

In two other patients (nos 4014 and 5639) and the father of deceased patient 6537, who displayed an identical band-shift on...
SSCP, sequence analysis revealed a T to C substitution, changing the Leu36 codon CTT into the proline codon CCT. In each case the normal sequence was visible as well. Family members of two cases were available to test the segregation of the Leu36Pro substitution. The mutation was identified in the mother and sister of patient 5639 (family A, Fig. 3A). Surprisingly, the sister had also inherited from the father the same allele of a COL4A3 CA-repeat (14; primer sequences available upon request) as the patient, but she lacked clinical features. In the second family (family B, Fig. 3A), we initially tested both parents, because the affected son (6537) had died at the age of 24 years. The Leu36Pro mutation had been identified in the healthy father (data not shown). But sequence analysis of DNA, extracted from paraffin-embedded hepatic tissue from the deceased son 6537, revealed that he had not inherited the Leu36Pro missense mutation from his father. His unaffected sister did not carry this mutation either. Both sibs inherited the same COL4A3 marker alleles from their parents (Fig. 3A). Subsequent clinical examination revealed proteinuria and haematuria in the mother, but not in the sister. This was confirmed by the segregation of marker S9 (DXS17; Fig. 3B), closely linked to COL4A5, making the X-linked form of Alport syndrome in this family more likely. Our data suggest that the Leu36Pro substitution is not the causative mutation in these families.

DISCUSSION
Pathogenic COL4A3 mutations
Three different pathogenic mutations have been detected in three unrelated patients with Alport syndrome from our sample of 22 cases. We have previously reported an affected brother and sister who were homozygous for a 5 bp deletion in the COL4A3 gene (14). In a second case the same 5 bp deletion was identified. In the third patient both COL4A3 genes contained premature stop codons (Arg43Stop and Ser86Stop). The identification of an Alport patient who was homozygous for the Arg43Stop mutation provided further support for the pathological significance of this mutation (14). Both nonsense mutations and the 5 bp deletion are predicted to cause COL4A3 chains in which more than 70% of the NC domain is absent (Fig. 2). It can now be safely concluded that the role of the COL4A3 gene in autosomal recessive Alport syndrome is evident.

In other disorders, caused by mutations in genes for structural proteins, both dominant and recessive mutations have been identified in the same gene (15–18). Mutations that prevent the formation or the incorporation of a protein are often recessive, whereas mutations that do not impair the appropriate targeting of extracellular proteins are usually dominant. The three mutations that we have identified in the COL4A3 gene introduce premature stop codons and will lead to a truncated protein. They will probably not be included in a collagen triple helical molecule, for which an intact NC domain is necessary (19). A recessive mode of inheritance is in line with these observations.

Non-pathogenic COL4A3 missense mutation?
In two patients and the father of a deceased patient a proline for leucine missense mutation was identified. The evidence that this amino acid substitution could cause Alport syndrome appeared to be strong. First, the specific leucine was conserved in all type IV collagen chains from Caenorhabditis elegans to humans (6, 20–27), which indicated functional significance. Furthermore, leucine is a strong helix-forming amino acid, whereas proline has helix-breaking properties (28). Computer analysis by protein modelling according to Chou-Fasman (29) predicted a change in hydrophobicity in the specific area due to the substitution (data not shown). Finally, we found the same Leu to Pro mutation in two unrelated patients with Alport syndrome and the father of a third patient. Therefore, we initially concluded that this mutation was involved in autosomal recessive Alport syndrome.

Segregation data in the family of two of these cases, however, were not in favour of a causative role. In family A (Fig. 3A) an unaffected sister of the male patient contained the same mutation in her maternal COL4A3 gene and inherited the same paternal COL4A3 CA-repeat allele as her brother. In case of autosomal recessive inheritance, she should have been affected as well. X-linked Alport syndrome was not very likely, because the mother and oldest sister carried the same COL4A5 allele as the affected boy (Fig. 3B), but lacked clinical signs. A new COL4A5 mutation in the male patient is an alternative explanation, but has still to be investigated.

Even more striking was family B (Fig. 3A), in which the healthy father carried the Leu36Pro substitution, but his affected son did not. This case was initially considered sporadic, but...
Table 1. Oligonucleotide primers used to amplify COL4A3 cDNA sequences (exons 1–5)

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<tr>
<th>Primer sequences</th>
<th>5'-CAG CTG CAT CAT GAT TGC C-3'</th>
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<tr>
<td>542: 5'-CAG CTG CAT CAT GAT TGC C-3'</td>
<td>551: 5'-GGA AAA CGT GGA GAC AGT GGA TC-3'</td>
</tr>
<tr>
<td>552: 5'-TAG AGA CCC AGA TCA CAG AAC TGA C-3'</td>
<td>543: 5'-ATA CCT AAA TAA CAG CAT GAT TGC C-3'</td>
</tr>
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Next, a detailed analysis of the text follows:

Further clinical investigations revealed haematuria and proteinuria in the mother, but not in the sister, making an X-linked mode of inheritance likely. This was confirmed by the segregation pattern of one of the marker DXS17 (Fig. 3B). From these data, it was evident that the Leu36Pro mutation was not causally involved in this family. Thus, without a functional assay, it is difficult to predict the effect of amino acid substitutions. Even drastic changes of strongly conserved amino acids, as in the Leu36Pro case, may not be pathogenic. The presence or absence of clinical features in an individual, who is homozygous for this mutation, will provide the definite answer to this question of pathogenicity. Whether this substitution can have a secondary effect on another mutated type IV collagen chain, with which it forms a triple helix, remains to be elucidated as well.

Genetic heterogeneity of Alport syndrome

The detection of pathogenic COL4A3 mutations in three patients with Alport syndrome in our sample is in line with previous studies, which suggested that about 10% of Alport syndrome pedigrees could be explained by autosomal recessive inheritance (8). Our group of 50 patients consisted of 28 clearly X-linked cases, in which we detected nine COL4A5 mutations after analyzing about 40% of the gene (30,31). In the remaining 22 cases, we have now identified three patients with a mutation in one or both of their COL4A3 genes. Therefore, about 6% of the total number of Alport syndrome cases (and about 13% of those that are not clearly X-linked) in our sample are caused by COL4A3 mutations. The actual number may be higher, because we only screened the five terminal exons of the COL4A3 gene (about 10% of the coding sequence). All cases with COL4A3 mutations had typical renal histology as well as characteristic high frequency hearing loss. Thus, the only obvious indication for an autosomal recessive aetiology was early-onset renal insufficiency in a female patient. In our initial studies we concentrated solely on COL4A5 as the major gene involved in Alport syndrome, but now we can conclude that analysis of the COL4A3 and COL4A4 (14) genes is warranted in autosomal recessive and sporadic Alport syndrome cases, especially if it concerns severely affected females.

MATERIALS AND METHODS

Alport patients

Twenty-two families with Alport syndrome, mainly of Dutch origin, were studied. Diagnosis was based on clinical course (main features: progressive renal failure and hearing loss, family history and the characteristic electron microscopic pattern in renal biopsies (32). DNA was extracted from blood (33) or from paraffin-embedded tissue (34).

SSCP analysis

PCR was performed on 100 ng leukocyte DNA in 25 μl standard mixture (Perkin-Elmer Cetus) using 50 ng primers in the presence of 2 μCi [α-32P]dCTP. Thirty cycles were performed consisting of 50 s at 92°C (denaturation), 50 s at 50°C (exon 4) or 55°C (exons 1, 2, 3 and 5) (annealing) and 1.5 min at 72°C (elongation). All five NC domain encoding exons could be amplified (14). SSCP analysis was performed on a 0.5×MDE-gel (AT Biochem, Malvern, PA) in 0.6×TBE buffer for 6 h at 40 W and for 16 h at 6 W at 4°C.

Sequence analysis

PCR fragments purified from agarose gels, using the ‘freeze squeeze’ method, were ‘cycle-sequenced’ (30) and analysed on an automated sequencer (Applied Biosystems 373A, Foster City, CA). Reaction conditions using dyeoxyterminators were according to protocols provided by the manufacturer (Applied Biosystems, Foster City, CA).

Polymorphic markers

CA repeat analysis was performed using a COL4A3/COL4A4 CA repeat marker according to a protocol developed by Mochizuki et al. (14; primer sequences and protocol available upon request). Markers S9 (DXS17) and COL4A5 (2B6) were tested as described before (36, 37).

RT–PCR analysis

Isolation of total RNA from peripheral blood lymphocytes and generation of COL4A3 cDNA was performed according to previously described methodology (30). Primer pairs 542/552 (outer) and 551/543 (inner) (Table 1) were used to amplify COL4A3 cDNA encompassing the NC domain. PCR fragments were isolated after electrophoresis by the ‘freeze squeeze’ method (31). Isolated CDNA fragments were cloned into pCR-ScriptTM(SK+) vector according to the manufacturer (Stratagene, La Jolla, CA). Individuals clones were isolated using standard procedures (38) and sequenced as described before.

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REFERENCES

