Albers-Schönberg disease (autosomal dominant osteopetrosis, type II) results from mutations in the ClCN7 chloride channel gene

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Albers-Schönberg disease, or autosomal dominant osteopetrosis, type II (ADO II), is the most common form of osteopetrosis, a group of conditions characterized by an increased skeletal mass due to impaired bone and cartilage resorption. Following the assignment of the gene causing ADO II to chromosome 16p13.3, we now report seven different mutations in the gene encoding the ClCN7 chloride channel in all 12 ADO II families analysed. Additionally, a patient with the severe, autosomal recessive, infantile form of osteopetrosis (ARO) was identified as being homozygous for a ClCN7 mutation. From genotype–phenotype correlations, it seems that ADO II reflects a dominant negative effect, whereas loss-of-function mutations in ClCN7 do not cause abnormalities in heterozygous individuals. Because some ARO patients have mutations in both copies of the ClCN7 gene, ADO II is allelic with a subset of ARO cases.

INTRODUCTION

In health, osseous tissue remodelling results from the balanced processes of bone formation and resorption. Excessive resorption causes osteoporosis, which accounts for most non-traumatic fractures (1). Conversely, defective bone resorption causes osteopetrosis characterized by dense but usually fragile bones. At least eight types of osteopetrosis have been described in humans (2). Deficiency of the carbonic anhydrase II isoenzyme causes autosomal recessive osteopetrosis (ARO) with renal tubular acidosis and cerebral calcifications (3; MIM 259730), whereas the majority of cases of malignant ARO (4; MIM 259700) reflect mutations in the TCIRG1 gene (5,6). Some rare cases of osteopetrosis, apparently with an autosomal recessive mode of inheritance, feature a milder phenotype and are therefore called the ‘intermediate’ form (7,8; MIM 259710).

Autosomal dominant osteopetrosis (ADO) is much more common than its recessive counterparts (9). However, due to its relatively benign clinical picture, with many patients being asymptomatic and only detected by coincidental radiographic examination, the prevalence of ADO is underestimated. Among families with ADO, two subtypes are generally reported based primarily on radiographic features (10,11). Type I (ADOI) features a generalized, diffuse osteosclerosis affecting especially the cranial vault (11). Type II (ADO II; MIM 166600), the form originally described in 1904 by Albers-Schönberg (12), is the most common form with an estimated prevalence of up to 5.5/100 000 (13). Clinical manifestations include non-traumatic fractures, especially of long bones, cranial nerve palsies, osteoarthritis of the hip and mandibular osteomyelitis (14). ADO II manifests radiographically with a segmentary osteosclerosis, predominately at

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the vertebral endplates (‘rugger jersey spine’), iliac wings (‘bone within bone’ sign) and skull base (9) (Fig. 1).

Recently, a genome-wide search led us to assign a gene underlying ADO II to chromosome 16p13.3 (15). Interestingly, the gene encoding the CICN7 chloride channel resides within the candidate region of 8.4 cM (16). In fact, the chloride channel is essential for the acidification of the extracellular resorption lacuna necessary for the osteoclast-mediated degradation of bone tissue (17), and is mutated in one patient with ARO (17). Therefore, we considered CICN7 both a positional and functional candidate gene for causing Albers-Schönberg disease.

RESULTS

Mutation analysis of ADO II families

Because the genomic sequence including the CICN7 gene is available from sequence databases (GenBank accession nos AL031600 and AL031705), mutation analysis of the exons and intron–exon boundaries is possible using genomic DNA. This effort disclosed seven distinctive mutations in CICN7 (Fig. 2) in 12 unrelated ADO II families (Table 1). Five mutations are missense, one results in a deletion of one amino acid and one deletes two nucleotides causing a frameshift affecting the C-terminal end of the protein. None of these mutations was found in 100 control chromosomes.

Four mutations are redundant mutations. The 2423delAG mutation was identified in a family from France and in an American family. The G215R mutation occurred in a French, a Danish and an American family. The P249L mutation was found in a French and a Danish family. Finally, the R767W mutation was identified in a French and an American family (Table 1). The different geographic origins of the families studied, as well as the analysis of microsatellite markers flanking the CICN7 gene (data not shown), indicate that these are independent mutations, i.e. not inherited from a common ancestor.

Mutation analysis of an ARO patient

Linkage analysis with markers from chromosome 16p13.3 clearly showed homozygosity for all markers in an ARO patient born from consanguineous, healthy parents (data not shown). Therefore, mutation analysis of CICN7 was performed demonstrating a homozygous missense mutation at position 766 (L766P) (Fig. 2). Both parents are heterozygous for this mutation.

Position and conservation of mutated amino acids

The transmembrane topology proposed for the CIC chloride channels suggests 10–12 transmembrane domains (18). The five amino acids involved in the missense ADO II mutations, as well as the mutation implicated in the ARO patient, are all highly conserved among the different members of the CIC chloride channel gene family (Fig. 3). The G215R and P249L mutations are recurrent mutations found in the patients of three and two unrelated families, respectively (Table 1). G215 is a highly conserved residue located between D2 and D3 (Fig. 4), a region known to influence the channels pore properties (19), whereas P249 takes part in a highly conserved, structural element forming a substantial part of the CIC channel pore (20). R286 is located in the extracellular space, just outside the
transmembrane domain D5. This amino acid is conserved among the different CIC proteins, with the exception of CIC1 and CIC2 which have a divergent sequence in this region of the protein. The two remaining ADO II mutations and the ARO mutation are in the cytosolic, C-terminal part of the protein involving neighbouring amino acids. G765, L766 and R767 are located within the D13 stretch, which coincides with the second CBS (cystathionine-β-synthase) domain described in the CLCN7 protein (21). All three mutations localize within the β2-strand of the CBS domain in which mutations have been reported to cause human disease (22). The precise function of this domain is still unclear, but a role in protein sorting has been suggested (21).

Finally, the two small deletions involve amino acids from the intracellular C-terminal part of ClCN7. Amino acid L688, deleted in one patient, is located between the two CBS domains, whereas the deletion of two nucleotides starting at nucleotide position 2423 results, in two unrelated families, in a protein that differs from wild-type ClCN7 only in the last 10 amino acids.

DISCUSSION

Osteopetroses in mammals comprise a heterogeneous group of conditions including at least eight different clinical entities in humans (2) and about nine spontaneous animal mutants (23). Furthermore, knockout mouse models for several genes cause osteopetrotic phenotypes, illustrating the diversity of factors involved in osteoclast differentiation and activation (23).

Recently, heterogeneity for human osteopetroses was underscored by genetic heterogeneity even within the ADO II subtype (24,25). This revelation was based on a linkage study involving an extended Danish family, which assigned the ADO II gene to chromosome 1p21 (26). However, this assignment was not confirmed in studies of other ADO II families. In

Table 1. Families included in the study

<table>
<thead>
<tr>
<th>Family</th>
<th>Phenotype</th>
<th>Origin</th>
<th>Mutation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>ADO II</td>
<td>France</td>
<td>2423delAG</td>
<td>15,34</td>
</tr>
<tr>
<td>B</td>
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<td>France</td>
<td>G215R</td>
<td>15,25</td>
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<td>Denmark</td>
<td>G215R</td>
<td>14,15,26</td>
</tr>
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<td>G</td>
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<td>–</td>
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<td>H</td>
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<td>31</td>
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<td>United States</td>
<td>L766P</td>
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</table>

Figure 2. (A) DNA and amino acid sequences neighbouring the seven different ADO II mutations. (B) DNA and amino acid sequences neighbouring the homozygous ARO mutation. The wild-type nucleotide and amino acid is given between brackets.
fact, in our recent linkage study, including six ADO II families, we localized the disease-causing gene on chromosome 16p13.3 and found in the same Danish family cosegregation between ADO II and a chromosome 16p13.3 haplotype (15). Therefore, we speculated that the evidence for linkage to chromosome 1p21 in this family reflected incidental cosegregation. In this current study, we confirmed this hypothesis by identifying a disease-causing mutation (G215R) in the *ClCN7* gene in this Danish family. This, and the fact that mutations in this gene were found in all 12 ADO II families analysed, suggests that ADO II is genetically homogeneous.

Our results also illustrate that ADO II is allelic with a subset of patients with the severe, autosomal recessive, infantile form of osteopetrosis. Previously, one ARO patient has been described as a compound heterozygote for a nonsense mutation (Q555X) and a missense mutation (R762Q) in the *ClCN7* gene (17). We now find a homozygous mutation (L766P) in another ARO patient.

Figure 5 illustrates our correlation between the different genotypes and phenotypes. As demonstrated by the phenotype of the CIC-7 knockout mouse, complete loss of function of the CIC-7 chloride channel causes severe osteopetrosis as seen in ARO patients. Because *CICN7* mutations have been characterized in only two ARO patients to date, it is impossible to know whether any phenotypical differences reflect the nature of the their mutations. Some rare cases of osteopetrosis have an ‘intermediate’ form. In these cases, an autosomal recessive mode of inheritance is proposed but the phenotype is milder than in ARO (7,8). Perhaps these cases are due to combinations of two mutations in *CICN7* that each only mildly reduce the capacity for Cl– conductance.

The ADO II mutations reported here are primarily missense mutations involving conserved amino acids (Figs 3 and 4). The two remaining mutations are small deletions that also preserve the major anatomy of the chloride channel, and therefore most likely result in effects comparable to the missense mutations. Because chloride channels seem to be organized as multimers, probably dimers (27), these mutations probably impair their function due to dominant negative effects. With such effects, most chloride channels will not function, explaining the phenotypical abnormalities.

In general, the parents of ARO patients are phenotypically normal. We assume that this reflects the fact that haploinsufficiency for this gene most likely do not cause clinical complications or radiographic findings. Alternatively, in some
cases this can be due to the reduced penetrance of ADO II. The parents of the ARO patient presented in this study do not have any clinical symptoms of ADO II, but a radiographic survey was not available.

The allelic nature of ADO II and ARO is supported in a report of an extended family segregating ADO II in which one individual manifested ARO (28). Perhaps in this family a mutated \textit{ClCN7} gene with a dominant negative effect causes ADO II which then coincides with a \textit{de novo} mutation, or a mutation inherited from the second parent causing ARO. The proposed explanation for both dominant and recessive forms of osteopetrosis associated with \textit{ClCN7} mutations parallels mutations in the \textit{ClC-1} gene causing myotonia. Mutations resulting in the loss of \textit{ClC-1} cause the autosomal, recessive form (Becker type) (29) whereas missense mutations manifest with the less severe, autosomal dominant form (Thomsen type) (30).

In conclusion, we show that most, if not all, cases of ADO II are caused by mutations in the \textit{ClCN7} gene. Based on the nature of the \textit{ClCN7} mutations, the ADO II phenotype probably results from a dominant negative effect. Our findings support the hypothesis that chloride channels generally act as homomultimers. Furthermore, our study illustrates the allelic nature of ADO II and a subset of ARO cases.

**MATERIALS AND METHODS**

**Families and patients**

Families A–F were previously described as they were used to localize the Albers-Schönberg disease gene on chromosome 16p13.3 (15). Samples G–L are from affected individuals from families with a history of Albers-Schönberg disease and several have also been described (31–33) (Table 1).

Family M lives in the United States but is of Chinese ancestry. The proposita is the child of a healthy couple and was born after a full-term gestation. The parents are second cousins. ARO was diagnosed at age 3 months when she presented to the New York Hospital Medical Center of Queens with Bell’s palsy. Radiographic skeletal survey revealed severe osteopetrosis and several non-displaced oblique fractures. She had anaemia, reticulocytosis, hepatosplenomegaly and mild optic nerve atrophy. The child underwent a mismatched allogeneic marrow transplant but died at the age of 18 months from sepsis and respiratory failure.

**Mutation analysis**

DNA was isolated from blood by standard procedures. Intron primers (Table 2), amplifying all coding exons and intron–exon boundaries of \textit{ClCN7}, were designed from genomic sequences (GenBank accession nos AL031600 and AL031705). The 25 exons were amplified from Genomic DNA Taq-polymerase or with PCR enhancer system (Gibco BRL), with enhancer concentration 1×. For all amplifications, 30 cycles were performed at a temperature specified in Table 2. The PCR products were purified with Concert Rapid PCR purification system (Life Technologies) and directly sequenced with the primers used for amplification, using BigDye terminator chemistry (Perkin-Elmer) on an ABI 3100 automated sequencer. Amplification of exon 9 resulted in a fragment of variable length because this exon follows a tandem repeat of a 50 bp sequence with variable copy number. In our set of samples the copy number varied between four and seven repeats. This repeat also interferes in some samples with the sequencing of this exon in the forward direction.

All exons in which putative mutations were detected, were sequenced in 100 control chromosomes without finding the mutations.

**ACKNOWLEDGEMENTS**

We thank the patients and families as well as the clinicians who provided material. This research was supported by a grant.
Table 2. Primers amplifying ClCN7 exons and intron-exon boundaries

<table>
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<th>Primers (5′–3′) reverse</th>
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