

## Original articles

## The *ALX4* homeobox gene is mutated in patients with ossification defects of the skull (foramina parietalia permagna, OMIM 168500)

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### Abstract

Foramina parietalia permagna (FPP) (OMIM 168500) is caused by ossification defects in the parietal bones. Recently, it was shown that loss of function mutations in the *MSX2* homeobox gene on chromosome 5 are responsible for the presence of these lesions in some FPP patients. However, the absence of *MSX2* mutations in some of the FPP patients analysed and the presence of FPP associated with chromosome 11p deletions in DEFECT 11 (OMIM 601224) patients or associated with Saethre-Chotzen syndrome suggests genetic heterogeneity for this disorder. Starting from a BAC/P1/cosmid contig of the DEFECT 11 region on chromosome 11, we have now isolated the *ALX4* gene, a previously unidentified member of the *ALX* homeobox gene family in humans. Mutation analysis of the *ALX4* gene in three unrelated FPP families without the *MSX2* mutation identified mutations in two families, indicating that mutations in *ALX4* could be responsible for these skull defects and suggesting further genetic heterogeneity of FPP.

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Foramina parietalia permagna (FPP), also referred to as the "Catlin mark" (OMIM 168500), is a skull ossification disorder characterised by ossification defects in the parietal bones (fig 1).<sup>1</sup> The size of the lesions is very variable among different patients, measuring from a few millimetres to several centimetres.<sup>2</sup> Although the openings decrease with time, surgical intervention may be required in severe cases to protect the underlying brain. In most cases, patients are asymptomatic, but the association of FPP with structural brain abnormalities or seizures has been described.<sup>3-5</sup> Recently, loss of function mutations in the *MSX2* homeobox gene have been established as the cause in some cases of FPP.<sup>6,7</sup> However, absence of *MSX2* mutations in some of the families analysed<sup>6,7</sup> and the presence of FPP in



Figure 1 Radiograph illustrating the presence of foramina parietalia permagna (white arrows) in a patient of family 3. X rays of patients of families 1<sup>6</sup> and 2<sup>12</sup> have previously been published.

other syndromes, such as DEFECT 11 (OMIM 601224) syndrome<sup>8</sup> or Saethre-Chotzen (OMIM 101400) syndrome,<sup>9</sup> suggests genetic heterogeneity for FPP.

The DEFECT 11 syndrome, also called deletion 11 contiguous gene syndrome as this is less controversial than the DEFECT 11 acronym, is caused by deletions in the proximal region of chromosome 11p. This syndrome is characterised by the presence of multiple exostoses (EXT), FPP, and sometimes additional features such as mental retardation and genital abnormalities.<sup>8</sup> Previously it has been shown that deletion of the *EXT2* gene is responsible for the development of EXT in these patients.<sup>10,11</sup> A second gene causing FPP must be located in close proximity to *EXT2*, as deletion analysis of several chromosome 11 deletion patients delineated a minimal candidate region for the FPP gene between the genetic markers D11S1393<sup>4</sup> and D11S2095.<sup>10</sup>

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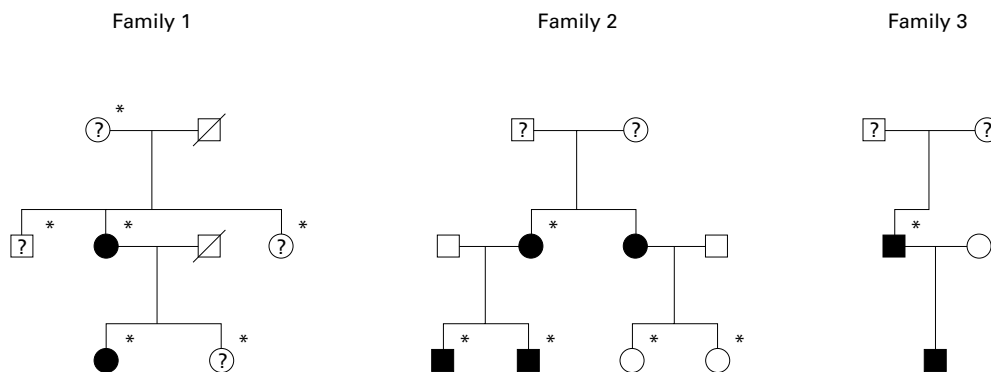


Figure 2 Pedigrees of the three FPP families analysed in this study. Filled symbols represent affected subjects, open symbols are unaffected subjects. Question marks indicate that the clinical status has not been confirmed by radiographs. DNA from subjects marked with an asterisk was available for analysis.

In an attempt to isolate the FPP causing gene involved in the DEFECT 11 syndrome on chromosome 11, we constructed a P1/cosmid/BAC contig of this region. By genomic sequencing combined with database searches and the use of exon prediction programs, we isolated the human orthologue of the *Alx4* gene from this candidate region and by mutation analysis we were able to show that some FPP patients harbour loss of function mutations in this gene.

### Patients and methods

#### PATIENTS

Six patients from three different families affected with FPP were included in this study (fig 2). Family 1 has already been described as family 4 in a previous publication describing the absence of a *MSX2* mutation in this family.<sup>6</sup> Family 2 originates from Italy and clinical details have been published previously.<sup>12</sup> The last FPP patient (family 3) is of Belgian origin. This patient was asymptomatic, but skull radiographs showed large defects in the parietal bones (fig 1). Diagnosis of all patients was based upon skull *x* rays.

#### PHYSICAL CHARACTERISATION OF THE FPP CANDIDATE REGION

A YAC/P1/cosmid contig of the DEFECT 11 candidate region on chromosome 11 has previously been constructed.<sup>10</sup> P1 clones ICRFP700D0494 and ICRFP700D0694 were digested with restriction enzymes *EcoRI*, *PstI*, *HindIII*, *DraI*, and *PvuII* and restriction fragments were ligated in PUC 18 vector. After transformation, subclones were sequenced using Big-Dye terminator chemistry (Perkin-Elmer) on an ABI 3100 automated sequencer and sequences obtained were aligned using the SeqManII (DNASTAR Inc) program. Additionally, BLAST searches against Genbank were performed with sequences obtained to identify overlapping clones in order to perform "contig walking".

#### IDENTIFICATION OF TRANSCRIBED SEQUENCES

Putative exons and transcribed sequences from the sequenced DEFECT 11 region were identified by analysis of the obtained contig sequence with the NIX program (<http://www.hgmp.mrc.ac.uk/Bioinformatics/>), which

Table 1 Primers amplifying *ALX4* exons

Exon	Primer (5' - 3')	Temp
1	aaactcccagccaaggcgcg aagccaagcaccctgtgcc ctctgtttggttcaaccattgg	65°C
2	tgcttaccagcctcactccc tccaggcggcatctcacc	60°C
3	ttctcagagcaccaggggctggg agggtcctctgggaagggcgag	65°C
4	agagcagagagtgggcggg	

includes GRAIL, Fex, Hexon, MZEF, Genemark, Genefinder, FGene, BLAST (against multiple databases), Polyah, RepeatMasker, and tRNAscan.

#### MUTATION ANALYSIS

Intron primers amplifying all coding exons and intron/exon boundaries of the *ALX4* gene were designed (table 1) and *ALX4* exons were amplified with PCR enhancer system (GIBCO BRL) with enhancer concentration 1×. For all amplifications, 35 cycles were performed at 65°C, except for exon 2 which was amplified at 60°C. Amplification products were purified with Concert Rapid PCR purification system (Life Technologies) and sequenced using Big-Dye terminator chemistry (Perkin-Elmer) on an ABI 3100 automated sequencer.

#### ASSAYS FOR THE DETECTION OF IDENTIFIED MUTATIONS

To confirm the 504delT mutation in family 1, PCR was performed with the PCR enhancer system (GIBCO BRL) with primers ALX4mut1 (5'-gccttctccaatgggctggg-3') and modified primer ALX4mod1 (5'-tgggtgagccagagtaccctc-3') at 55°C and 1× enhancer concentration. The use of the ALX4mod1 primer creates a new *Taq* restriction site in the mutant amplification product. After digestion with *TaqI* restriction enzyme at 65°C for two hours, products were analysed on 12% acrylamide gels. The presence of the 504delT mutation results in the digestion of the mutant 132 bp fragment in fragments of 111 bp and 21 bp.

The presence of the G815C mutation in family 2 and controls was tested by reamplification of exon 3 followed by *MspI* restriction digest and analysis of the digested products on a 12% polyacrylamide gel. Presence of the C

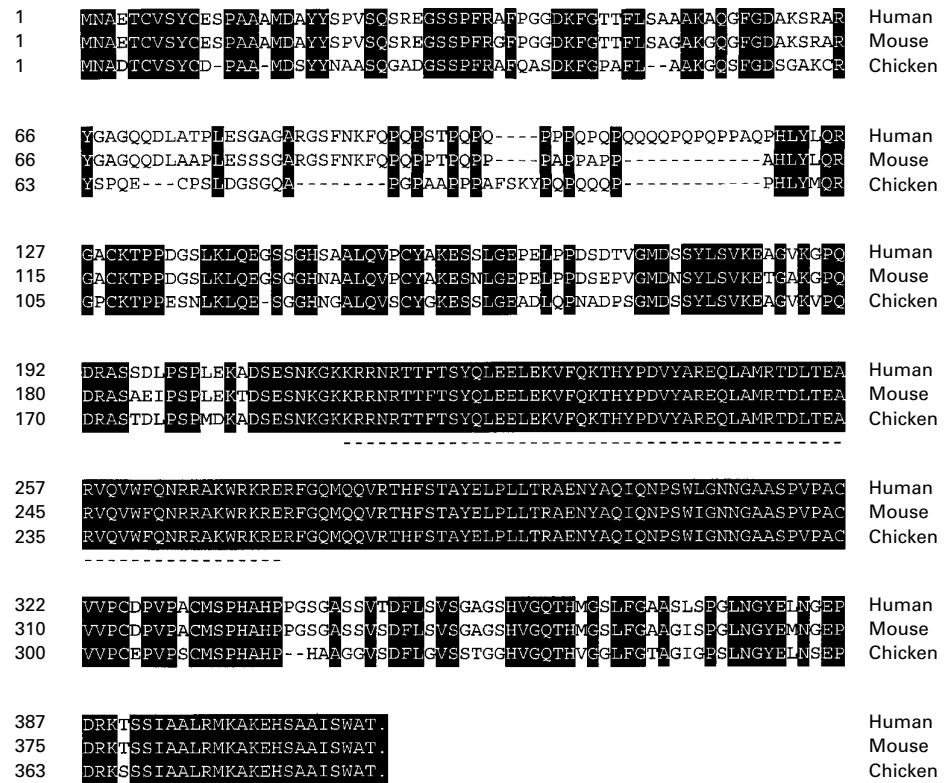


Figure 3 Alignment of the *ALX4* proteins of human, mouse, and chicken. Amino acids conserved in the three species are represented in black boxes. The 100% conserved homeodomain is underlined.

allele results in digestion of the 234 bp amplification product in bands of 147 bp and 87 bp.

The G729A polymorphism was characterised by amplification of exon 2 followed by *CfoI* restriction digest. The 400 bp amplification product is digested in bands of 311 bp and 89 bp in the presence of the G allele. The presence of the A allele results in absence of the *CfoI* restriction site yielding a 400 bp band only. All digested products were analysed on 2% agarose gels.

## Results

### IDENTIFICATION OF THE *ALX4* GENE

In order to isolate a gene responsible for FPP we concentrated on the proximal region of chromosome 11p, which is known to be deleted in patients suffering from the chromosome 11 deletion contiguous gene syndrome. From a previously constructed YAC/P1/cosmid contig of this region we selected P1 clones ICRFP700D0494 and ICRFP700D0694, known to contain the 5' and 3' ends of the *EXT2* gene respectively.<sup>10</sup> Sequences from both P1 clones were obtained by direct sequencing on subclones generated by restriction digestion of the P1 clone with five different enzymes followed by ligation of the fragments in PUC18 vector. Overlapping clones were identified by sequence alignment. Aligned sequences were blasted against sequence databases, including the unfinished sequences from the Human Genome Project. At the distal side of the candidate region, P1 clone ICRFP700D0494 overlaps with PAC clone pDJ404m15 (AC 002554), containing 127 590 bp of contiguous

sequence, including distal flanking marker D11S1393. At the proximal side, two additional genomic clones, 706A13 (AC 0119143) and 70A24 (AC 025533), were identified which partially overlapped ICRFP700D0694. The working draft of chromosome 11 clone RP11-706A13 consisted of 28 unordered pieces of a total of 190 737 bp, while the available sequence of clone 70A24 consisted of 25 unordered pieces covering 167 817 bp. Combined sequence information of all clones allowed us to construct a sequenced contig of the DEFECT 11 region with only a few remaining gaps. Sequences from these contigs were further analysed for the presence of known genes or ESTs and putative exons using the NIX program. No known genes, except for the *EXT2* gene, were found to be present in this region, but several ESTs and predicted exons were identified. Four exon predictions were found to show high sequence homology to the mouse *Alx4* gene,<sup>13</sup> suggesting that they represent the human *ALX4* gene. Sequence analysis showed that the entire *ALX4* coding region is contained in these four exons, which contain respectively 466, 311, 129, and 330 bp of coding sequences. They encode a protein consisting of 411 amino acids, showing an overall identity of 92.5% with its mouse counterpart and 78.9% with chicken *Alx4*. The identity in the homeobox region, which is spread over exons 2 and 3, is 100% and remains high in the carboxy-terminal region (fig 3). Sequences were submitted to Genbank (Accession numbers AF308822 to AF308825).

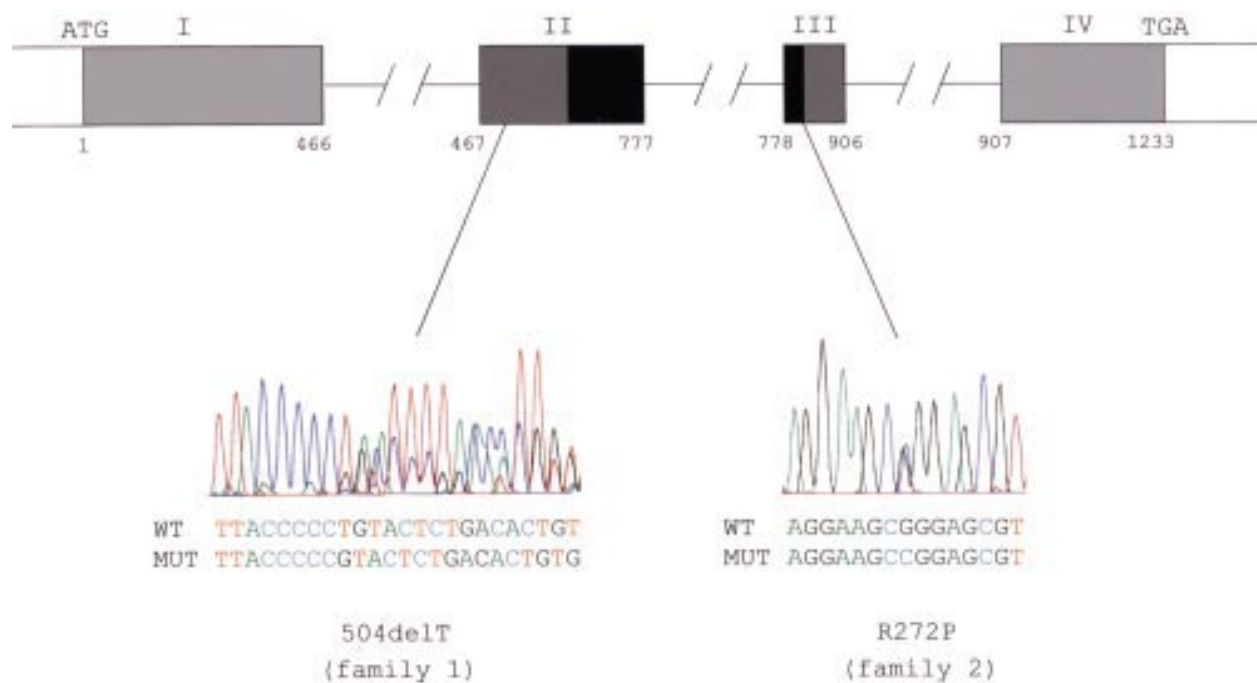


Figure 4 Mutations in the *ALX4* gene in our FPP patients. The four *ALX4* exons are represented with the coding area in grey and the homeodomain in black. Corresponding cDNA positions are indicated at the beginning and end of each exon. Electrophoretograms with both wild type (WT) and mutated (mut) sequence of the two different mutations in family 1 (504delT) and family 2 (R272P) are shown and the position of each mutation in the *ALX4* gene is indicated.

#### MUTATION ANALYSIS

We ascertained DNA from six FPP patients belonging to three different families. In all patients, the *MSX2* gene, previously shown to be mutated in a fraction of FPP patients<sup>6,7</sup> was excluded as the disease gene by sequencing the entire *MSX2* coding region and splice junctions.

Primers amplifying all four *ALX4* exons were designed (table 1) and the entire coding region and intron/exon boundaries were sequenced in one patient from each family. In family 1, a deletion of a thymidine was found in exon 2 (fig 4) at position 504 of the cDNA (adenosine of start codon +1). This mutation results in a premature stop codon after 179 amino acids. To confirm this mutation, we designed a modified primer (*ALX4mod1*) creating a *TaqI* restriction site in the presence of the mutation and we analysed all available family members by a PCR/restriction assay. This confirmed the presence of the 504delT mutation in all the patients of this family. In family 2, a guanosine to cytosine substitution at cDNA position 815 (adenosine of start codon +1) results in a R272P missense mutation (fig 4). As the mutation creates a *MspI* restriction site, we analysed the remaining family members and controls for the presence of this mutation by amplification of exon 3 followed by *MspI* restriction digest. All affected family members showed the mutation specific restriction pattern, which was not detected in the healthy family members nor in any of the 50 controls. In the only available patient from family 3, a 729G→A substitution in exon 2 was identified. However, this substitution does not alter the amino acid sequence (A243A) and represents a common polymorphism. Analysis

of 100 control chromosomes showed that the G allele is present on 85% of the chromosomes and the A allele on 15%. We could not detect any other sequence variation in the *ALX4* gene in this patient.

#### Discussion

Chromosomal rearrangements have often provided crucial information about the precise localisation and identification of disease causing genes. Even more information can be gained from contiguous gene syndromes, where comparison of deletions in different patients provides data on the relative mapping of several genes. The DEFECT 11 or deletion 11 contiguous gene syndrome is caused by deletions in the proximal region of chromosome 11p, which result in a clinical phenotype including multiple exostoses (EXT), FPP, and, depending on the extent of the deletion, additional features such as mental retardation and genital abnormalities.<sup>4,8</sup> This syndrome has already been helpful in the identification of the *EXT2* gene,<sup>10</sup> which is responsible for the development of multiple exostoses, but also provided evidence for the presence of a gene causing FPP in this chromosomal region. Therefore, it was not surprising that when the *MSX2* gene was recently proven to be causative for FPP,<sup>6,7</sup> only a proportion of the patients harboured a mutation in this gene.

Our efforts towards the identification of the FPP gene on chromosome 11 were mainly focused on genomic sequencing and the in silico identification of transcribed segments from the sequences obtained. Among the various exon predictions and ESTs identified, four putative exons were considered to be of interest, since they comprised a deduced open

reading frame coding for a 411 amino acid protein with 92.5% identity to the mouse *Alx4* gene. Because of this high degree of identity throughout the whole open reading frame and the fact that alignment of the amino acid sequence to the mouse and chicken *ALX4* shows that both start and stop codon positions are present, we are confident that the 411 amino acid protein represents the full length human *ALX4* (fig 3).

The *ALX* transcription factors belong to a family of genes that are related to the *Drosophila* gene *aristaleless*. Members of this homeobox family can bind palindromic DNA sequences as a homodimer or heterodimer with other family members.<sup>14,15</sup> Previously, expression studies of the mouse *Alx4* gene suggested that the *Alx4* protein plays an important role in the patterning of structures derived from the craniofacial mesenchyme, the first branchial arch, and the limb bud.<sup>13,16</sup> Further functional studies have shown that a negative feedback loop between *ALX4* and *Sonic hedgehog* (*Shh*) exists, which is required for proper establishment of anteroposterior polarity during vertebrate limb development.<sup>17</sup> *Alx4* knockout mice show several abnormalities, including polydactyly and ventral body wall defects, but most interestingly a decreased size of the parietal plate of the skull,<sup>17,18</sup> which was the main reason for considering the *ALX4* gene as a strong candidate gene for FPP.

Mutation analysis of the *ALX4* gene showed two different mutations in our FPP families. In family 1, an inactivating frameshift mutation (504delT) was found, indicating that haploinsufficiency of the *ALX4* gene results in FPP. This is not unexpected, as already illustrated by the DEFECT 11 syndrome being caused by heterozygous deletions in the 11p region.<sup>8</sup> Interestingly, in family 2, a R272P missense mutation was found, which was not detected in any of the controls. Arginine 272 belongs to the homeobox domain which is 100% conserved between human, mouse, and chicken (fig 3), illustrating its high functional importance. Moreover, arginine 272 is also conserved in the other family members *ALX3* and *CART1*. It is a component of the recognition helix 4 of the homeodomain<sup>19</sup> and substitution of arginine for proline is likely to disrupt DNA binding also leading to a loss of function. The identification of *ALX4* mutations in two of our FPP families now confirms the genetic heterogeneity of FPP. Taking together all the data,<sup>6,7</sup> a *MSX2* mutation was found in seven out of 16 families analysed. From the remaining nine families, we analysed three, resulting in a mutation in two of them. This suggests that mutations in both genes are responsible for the majority of FPP cases.

We could not detect any mutation in either gene in the FPP patient of family 3 presented in this study. It may still be that the presence of FPP in this patient is the result of a mutation in intronic or regulatory sequences outside the *ALX4* or *MSX2* coding region, which we did not analyse. Alternatively, it is possible that this

patient illustrates further genetic heterogeneity of FPP.

Based on the restricted patient population currently available, no evidence can be found for a genotype-phenotype correlation between mutations in the *MSX2* and *ALX4* genes. The fact that haploinsufficiency of both genes results in identical phenotypes indicates a still unknown functional link between these two transcription factors. Characterisation of this link and elucidation of the complete pathway involved will provide further insights into the ossification process of the skull.

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