

ONLINE MUTATION REPORT

Familial expansile osteolysis in a large Spanish kindred resulting from an insertion mutation in the *TNFRSF11A* gene

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Familial expansile osteolysis (FEO) is a rare autosomal dominant disorder resembling Paget's disease of bone (PDB), characterised by osteolytic lesions. These are mainly located in the long bones and spare the axial skeleton. Progressive osteoclastic resorption accompanied by medullary expansion leads to severe, painful, disabling deformity and a tendency to pathological fracture. Characteristically, FEO is accompanied by deafness and loss of dentition as a result of middle ear and jaw abnormalities, and biochemically serum alkaline phosphatase levels are variably raised.¹⁻³ FEO cases present with appendicular lesions,⁴ while PDB patients tend to present with trunk and skull lesions.⁵

The FEO gene was mapped to 18q21.1-q22 by linkage to DNA markers⁶ and recently Hughes *et al*⁷ have identified mutations in the gene encoding the receptor activator of nuclear factor-kappa-B (RANK, *TNFRSF11A*) as a cause of FEO. They proceeded to identify two different heterozygous insertion mutations in exon 1 of the *TNFRSF11A* gene in affected members of four families with FEO or familial PDB. All mutations affected the signal peptide region of the RANK protein. Expression of recombinant forms of the mutant RANK proteins showed alterations in expression levels and lack of normal cleavage of the signal peptide. Both mutations caused an increase in RANK mediated nuclear factor-kappa-B signalling in vitro, consistent with the presence of an activating mutation. Although PDB and FEO share some clinical features (bone resorption, deafness, and loss of dentition), linkage to 18q21.1-q22 is unusual in familial PDB and three other candidate loci have been described in PDB kindreds, indicating the genetic heterogeneity of this disease.^{7,8} There are some rare early onset PDB cases which show *TNFRSF11A* mutations, but in the vast majority of cases, no mutation is found.⁹ Recently, Whyte *et al*^{10,11} described a 15 base pair duplication in the *TNFRSF11A* gene in a mother and a daughter affected by expansile skeletal hyperphosphatasia (ESH), characterised by early onset deafness, premature loss of teeth, hyperostotic widening of the long bones, and accelerated remodelling. Clinically, this was not considered a variant of FEO but the insertion found shows that ESH and FEO are allelic.

Here we report a large kindred from eastern Spain spanning four generations with 20 members affected. All affected subjects harbour the 18 bp insertion in exon 1 reported in the first study.⁹ This is the first genetic characterisation of a FEO family after the original description of mutations in FEO and confirms that exon 1 could be a hot spot for mutational events in the RANK gene.

MATERIAL AND METHODS

Patients

The pedigree of the family is shown in fig 1. Fourteen members of the family were involved in the study and the pattern of inheritance was consistent with an autosomal

dominant disorder. Clinical diagnosis of FEO was suspected, based on a clinical history of bone pain and deformities of the long bones. All affected subjects had biochemical, dental, and hearing abnormalities and radiological examination showed the presence of osteoporosis in subjects from generations II and III. Subjects without the mutation appear in the pedigree as unaffected, regardless of some possible clinical signs. In table 1 we show the last clinical evaluations of 22 members of the pedigree.

II.4

An x ray examination showed a single osteolytic lesion in the medial third of the radial diaphysis (size 5 × 3 cm), without

Key points

- Familial expansile osteolysis (FEO) is an autosomal dominant disorder, similar to Paget's disease, characterised by osteolytic lesions, deafness, and dental alterations. Recently, mutations in the *TNFRSF11A* gene, encoding the receptor activator of nuclear factor-kappa-B (RANK), have been identified as the cause of FEO.
- In this study we describe the molecular characteristics of a large, four generation Spanish family with 20 members affected by FEO. After the study of 14 subjects, although initial features suggested Paget's disease, later clinical examinations clearly excluded this entity as the cause of the disease in this family.
- After PCR amplification, polyacrylamide gel electrophoresis and direct sequencing, we found the same 18 bp duplication in exon 1 of the *TNFRSF11A* gene identified in the two families of the original study, and we show that the insertion mutation is stable throughout all meiotic cycles.
- These results suggest that exon 1 of the *TNFRSF11A* gene is a hot spot for mutations and that this duplication tends to be stable and presents with phenotypic heterogeneity. Specific analysis of exon 1 should be considered as a method of genetic diagnosis as well as an approach to counselling in further affected families, especially in young subjects and those with doubtful clinical signs.

Abbreviations: FEO, familial expansile osteolysis; PDB, Paget's disease of bone; ESH, expansile skeletal hyperphosphatasia; RANK, receptor activator of nuclear factor-kappa-B

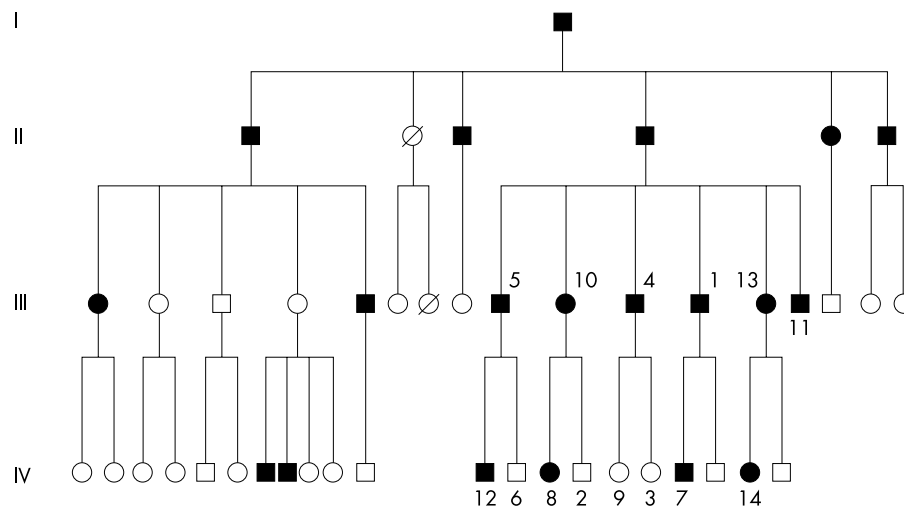


Figure 1 Pedigree of the family. Numbers indicate the subjects analysed for the 18 bp insertion. II.2 and III.7 have died. In some cases with just dental alterations, the subjects have been considered provisionally unaffected. Even though IV.17 (number 3) appeared to have some signs of deafness, she did not present with the mutation. Sometimes, subjective clinical evaluations can result in mistakes in diagnosis. Deafness and dental alterations present some difficulties when diagnosing some subjects.

sclerotic reaction (fig 2). An anatomopathological examination of a radial diaphysis and epiphysis sample was performed. Macroscopically, an irregular surface, a hard consistency, and a brown colouration were observed. Microscopically, the bone tissue of cortical origin showed a lamellae and trabecular structure with a marked increase of bone remodelling and medullary fibrosis. Thus, we can exclude fibrous hyperplasia, osteogenesis imperfecta, and hyperostosis. The presence of large amounts of large osteoclasts, with a minimum of 11 nuclei, made it possible to consider Paget's disease. Nevertheless, subsequent clinical evaluations differentiate this entity from PDB. We could only find destructive focal lesions in one other patient (III.10, daughter of II.4), the radius again being the affected bone. In this case, the lesion was mainly expansive, with possible microlytic lesions but not clearly osteolytic. In the description of Osterberg *et al.*,¹ affected subjects presented with a much higher number of focal lesions. All affected members of the pedigree showed marked osteoporosis on densitometry, with no clear

osteolytic lesions. However, the osteoporotic alteration of the trabecular network may result, in the future, in osteolytic lesions. X ray examination showed that bones present with a clear aspect, decalcified and radiotransparent, resembling crystal. The gammagraphy images showed a focal activity during reheating episodes, typically in the wrists, knees, and ankles, not seen radiologically. Focal lesions are more often found in long bone diaphyses, sparing articular areas. In PDB we would more frequently expect the vertebral spine, pelvis, sacral bone, and skull to be affected. In PDB there is a thickening of bone cortex and hypertrophy, which is the opposite to what happens in FEO. Also in PDB, the skull is dense and heterogeneous. Radiological and tomodensitometric examinations of the skull did not show any alteration but a mild demineralisation or complete disappearance of the ossicular chain, depending on the stage of the disease. No cochlear demineralisation was observed. There was an important hyperpneumatisation of the mastoids, already apparent at an early age. Later radiological examinations (20 years after) showed no evident change. In most patients, the second clinical manifestation, after deafness, is dental, shown as an apical and/or cervical resorption, resulting in an early loss of teeth. There was neither bone osteitis nor periodontal osteolysis, and the lamina dura was conserved, unlike in PDB.

Table 1 Current clinical evaluation of some members of the pedigree

| Subject | Age at onset | Deafness | Dental alterations | Bone alterations |
|---------|--------------|----------|--------------------|------------------|
| II.1 | 12 | +++ | +++ | +++ |
| II.3 | 12 | +++ | +++ | +++ |
| II.4 | 11 | +++ | +++ | +++ |
| II.5 | 13 | +++ | +++ | +++ |
| II.6 | 13 | +++ | +++ | +++ |
| III.1 | 35 | - | + | + |
| III.5 | 12 | - | + | + |
| III.9 | 20 | ++ | + | - |
| III.10 | 20 | ++ | ++ | ++ |
| III.11 | 11 | + | +++ | + |
| III.12 | 15 | ++ | +++ | ++ |
| III.13 | 10 | ++ | ++ | + |
| III.14 | 10 | + | + | + |
| III.16 | 15 | - | + | - |
| IV.1 | 17 | - | + | - |
| IV.3 | 12 | - | + | - |
| IV.4 | 13 | - | + | - |
| IV.12 | 12 | +++ | - | - |
| IV.14 | 11 | +++ | - | - |
| IV.17 | 11 | + | - | - |
| IV.18 | 10 | + | - | - |
| IV.20 | 7 | +++ | - | - |

Molecular genetic studies

Genomic DNA was extracted from leucocytes by previously described methods.¹² As in the four previously reported cases mutations were found in exon 1, we proceeded to study directly that area of the gene. The coding sequence of exon 1



Figure 2 Radiological image of the radius from patient II.4. The long arrow shows an isolated osteolytic lesion without sclerotic reaction. The short arrows show the generalised alteration of the trabecular network.

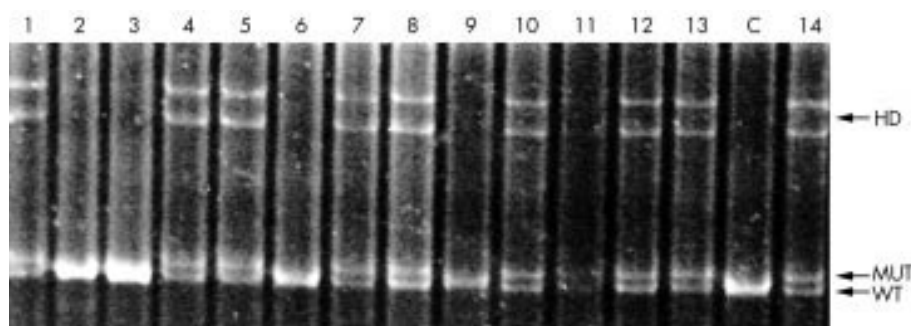


Figure 3 Acrylamide gel electrophoresis showing the different patterns of affected and unaffected subjects: 2, 3, 6, 9 and C (control) are unaffected; 1, 4, 5, 7, 8, 10, 11, 12, 13, and 14 are affected. HD: heteroduplexes, MUT: mutant allele with the 18 bp insertion, WT: 534 bp wild type allele.

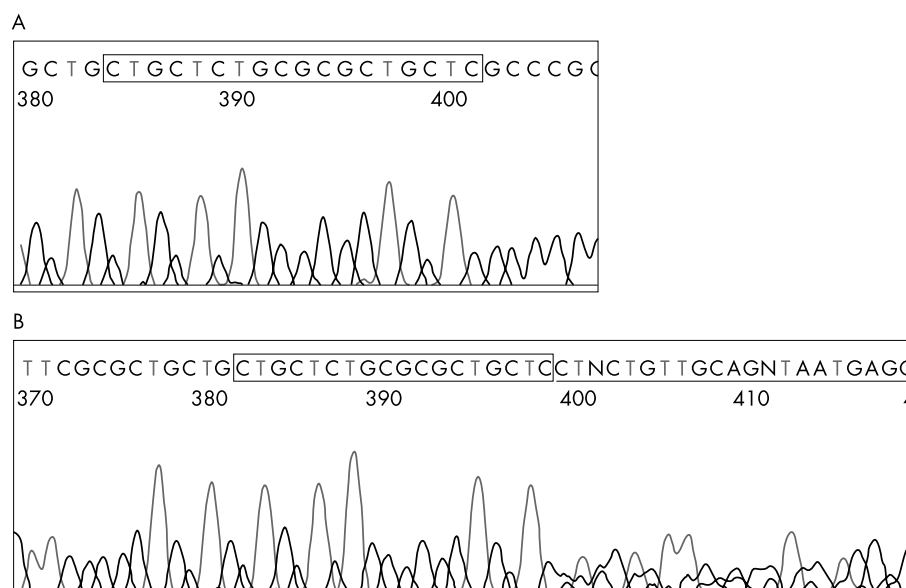


Figure 4 18 bp FEO mutation. Sequence electropherograms of a patient and a control. (A) Normal control sense strand. (B) FEO mutant sense strand. The 18 bp duplicated sequence is boxed.

was amplified by PCR using appropriate primers.⁵ PCR conditions were performed in a final volume of 50 μ l using the following conditions: initial denaturation at 95°C for five minutes followed by 30 cycles of 94°C for one minute, annealing at 65°C during the first 10 cycles, then 63°C for one minute, and extension at 72°C for one minute. PCR products were purified and sequencing reactions were done using the Big-dye™ (Perkin Elmer Applied Biosystems) terminator kit in an ABI-PRISM™ 310 automatic sequencer (Perkin Elmer Applied Biosystems).

To detect the presence of the mutation in all subjects studied, PCR products were amplified and run on a 5% polyacrylamide gel. The gel was stained with ethidium bromide and mutant alleles were confirmed by size variation and the presence of heteroduplexes of the mutant and wild type PCR fragments.

RESULTS

Fig 1 shows the pedigree of the family. Fourteen subjects from three different generations were analysed to determine whether patients in this family were carrying the recently defined mutation in the *TNFRS11A* gene.⁵ Amplification of a 534 bp fragment of exon 1 and further PAGE analysis of amplicons showed that all clinically affected members displayed size variations compatible with the reported 18 bp duplication of bases 84-101 in exon 1. Fig 3 shows the presence, in the affected subjects, of the two bands

corresponding to the wild type and mutated bands as well as heteroduplex wild type/mutation. In the unaffected subjects, only the expected wild type band can be seen. Sequence analysis of the amplified products confirmed the expected insertion and showed that it occurred in the same position as previously reported (fig 4).

To study a putative instability of the duplication during meiotic cycles, five subjects from different generations (II.4, IV.12, IV.14, IV.18, and IV.20) were studied. These subjects represent a total of 10 meiotic divisions from generation II to generation IV. PCR and sequencing analysis showed that in these patients the insertion was the same size (data not shown).

DISCUSSION

The gene encoding receptor activator of nuclear factor-kappa-B (RANK), *TNFRS11A*, has been identified as that responsible for FEO.⁵ These authors studied the genetic basis of FEO in the same family where linkage to chromosome 18 was established,⁶ and in an unrelated American family. A male from a German family diagnosed with osteolytic expansile PDB was also included. Interestingly, Cody *et al*³ were also able to link PDB to the FEO locus, suggesting that Paget's disease and FEO were allelic versions of the same disorder. However, different studies of series of patients with both familial or sporadic PDB (one of them from Spain) did not identify mutations in the *TNFRS11A* gene,^{7-9 14-16} suggesting that other

genes situated in the same or other chromosomal loci could be involved in the pathogenesis of this disorder, as in many cases linkage of PDB to 18q is not clear. In fact, recently, Hocking *et al*⁸ found three candidate loci for PDB on 2q36, 10p13, and 5q35.

We were able to characterise the clinical traits of affected members of different generations and ages. In our family, the natural history of FEO seems to follow a regular pattern. Usually, the first clinical sign is deafness, which appears between 7 and 12 years of age. Later, during the second decade of life, patients develop loss of dentition and finally the typical bone alterations may appear. This pedigree shows the extent of intrafamilial genetic variability as some affected subjects (III.1, III.5, III.16, IV.1, IV.3, and IV.4) presented with dental alterations at onset and at present they do not show deafness (table 1). In this family, bone alterations appeared when dental and auditory problems were already evident. At the beginning, after the first biopsy examination, PDB could not be excluded, but the subsequent clinical evaluations did exclude it. Only II.4 and his daughter III.10 presented with destructive bone lesions in the radius, being clearly osteolytic in II.4 and expansive in III.10; the rest of the affected subjects in the pedigree presented with demineralisation. Thus, the entity in this family was microscopically similar to PDB (increased osteoclastic activity) but radiologically, clinically, and macroscopically different from it. On the other hand, pathological fractures, which are common in FEO, did not occur, even in those from generation II (between the fourth and fifth decades of life). These differences in the presentation of the phenotype in patients harbouring the same molecular defect strongly suggest a certain degree of phenotypic variability for the same mutation, which should be taken into account when a clinical diagnosis of FEO is suspected.

The recently described ESH shares some clinical features with FEO but large osteolytic lesions are absent. However, the 15 bp insertion found in *TNFRSF11A* makes ESH an allelic variant of FEO.¹⁰ Comparing the three entities, we could conclude that FEO and ESH are very close and become allelic variants at 18q21.1-q22 and both differ from classical PDB mainly in the sparing of the axial and skull bones, even when signs of hyperostosis and osteosclerosis in the skull are detected.¹¹ The difference between ESH and FEO is the absence of large osteolytic lesions in the former.¹⁰ In our kindred, differing from previously described FEO families, we found just two affected subjects with clear lesions, in each case involving only one bone.

Hughes *et al*⁹ identified a tandem duplication of bases 84-101 of exon 1 in two unrelated families, while the duplication was not found in the unaffected subjects, in 90 subjects with sporadic PDB, or 158 controls. The same duplication was also found in the only available member of a small family of German origin. In addition, a study of four families with PDB showed, in one person, the presence of a larger duplication involving bases 75-101 in exon 1 that cosegregated with the disease. These findings prompted us to study a putative instability of the duplication as the pedigree contains a large number of meiotic divisions. However, PCR and sequence analysis data showed that the duplication is stable throughout meiosis and its presence does not predispose to a further genetic instability.

According to these findings, it seems that the 18 bp in frame insertion within the signal peptide region of RANK could be a common mutation of FEO and that this mutation by itself may present with some phenotypic variability. The results found in

the Spanish family reported here, which to our knowledge constitutes the first one since the identification of the gene, strongly support the concept that the 18 bp tandem duplication of exon 1 constitutes a hot spot that will facilitate genetic diagnoses and counselling in FEO affected families.

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