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ORIGINAL ARTICLE

Elahe Elahi · Yousef Shafaghathi · Sareh Asadi
Farnaz Absalan · Hani Goodarzi · Nava Gharaii
Mohammad Hassan Karimi-Nejad · Farhad Shahram
Anne E. Hughes

Intragenic SNP haplotypes associated with 84dup18 mutation in *TNFRSF11A* in four FEO pedigrees suggest three independent origins for this mutation

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Abstract Familial expansile osteolysis (FEO) is a rare disorder causing bone dysplasia. The clinical features of FEO include early-onset hearing loss, tooth destruction, and progressive lytic expansion within limb bones causing pain, fracture, and deformity. An 18-bp duplication in the first exon of the *TNFRSF11A* gene encoding RANK has been previously identified in four FEO pedigrees. Despite having the identical mutation, phenotypic variations among affected individuals of the same and different pedigrees were noted. Another 18-bp duplication, one base proximal to the duplication previously reported, was subsequently found in two unrelated FEO patients. Finally, mutations overlapping with the mutations found in the FEO pedigrees have been found in ESH and early-onset PDB pedigrees. An Iranian

FEO pedigree that contains six affected individuals dispersed in three generations has previously been introduced; here, the clinical features of the proband are reported in greater detail, and the genetic defect of the pedigree is presented. Direct sequencing of the entire coding region and upstream and downstream noncoding regions of *TNFRSF11A* in her DNA revealed the same 18-bp duplication mutation as previously found in the four FEO pedigrees. Additionally, eight sequence variations as compared to the *TNFRSF11A* reference sequence were identified, and a haplotype linked to the mutation based on these variations was defined. Although the mutation in the Iranian and four of the previously described FEO pedigrees was the same, haplotypes based on the intragenic SNPs suggest that the mutations do not share a common descent.

E. Elahi (✉) · S. Asadi · F. Absalan
National Institute for Genetic Engineering and Biotechnology,
Tehran-Karaj Expressway, Km 17 Pajouhesh Boulevard, Tehran,
Iran
Tel. +98-21-22181251, +98-21-44580344, +98-21-22547464;
Fax +98-21-44580399
e-mail: elahe.elahi@acnet.ir, elahe.elahi@kahyam.ut.ac.ir, elahe.elahi@gamil.com

E. Elahi · S. Asadi · F. Absalan
Dept. of Biological Sciences, Faculty of Science, University of
Tehran, Tehran, Iran

E. Elahi
Bioinformatics Center, Institute of Biochemistry and Biophysics,
University of Tehran, Tehran, Iran

Y. Shafaghathi
Genetics Research Center, University of Welfare Science and
Rehabilitation at Evin, Tehran, Iran

H. Goodarzi · N. Gharaii
Dept. of Biotechnology, Faculty of Science, University of Tehran,
Tehran, Iran

M.H. Karimi-Nejad
Karimi-Nejad/Najmabadi Genetics Center, Tehran, Iran

F. Shahram
Rheumatology Research Center, Tehran University of Medical
Sciences, Tehran, Iran

A.E. Hughes
Dept. of Medical Genetics, The Queen's University of Belfast,
Belfast, UK

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Introduction

Familial expansile osteolysis (FEO; OMIM 174810) is a rare autosomal dominant disorder causing bone dysplasia. The disease was first described in 1976 [1]. Its features include early-onset hearing loss, often in childhood, tooth destruction, and progressive lytic expansion within limb bones causing pain, fracture and deformity. Its radiographic and histopathological focal skeletal defects initially resemble those observed in the active osteolytic phase of the far more common Paget's disease of bone (PDB), but evolve to expanded shell-like bones that are fat filled and almost totally devoid of bony cortex and trabecular bone pattern [2]. Defects in PDB generally affect the axial skeleton, but lesions in FEO are mostly appendicular [3].

Five families afflicted with FEO and one sporadic case of FEO have been described [4–6]. The largest of these pedigrees was from Northern Ireland, and the others were from Germany, the United States, and Spain. The disease was originally linked to chromosome 18 in the Irish family

and, later, *TNFRSF11A* was identified as the causative gene in all the familial and sporadic cases [4–7]. *TNFRSF11A* encodes receptor activator of nuclear factor- κ B (RANK) and was considered an appropriate candidate gene because RANK is expressed on osteoclast precursor cells and is a receptor for RANK ligand-mediated signaling affecting osteoclast formation, differentiation, and function [8]. An identical in-frame 18-base pair (18-bp) tandem duplication (84dup18; g.84_101dupCTGCTCTGCGCGCTGCTC; c.46_63dupCTGCTCTGCGCGCTGCTC) in the first exon of *TNFRSF11A*, affecting the signal peptide region of RANK, was identified as the disease-causing mutation in four of the families [4,5]. The mutation existed in the heterozygous state in affected individuals. Expression level and cleavage of the signal peptide of recombinant RANK carrying the mutation were shown to be perturbed. Functionally, the mutation caused increased RANK-mediated nuclear factor- κ B signaling in vitro [4]. The mutation in the remaining familial and sporadic cases was another 18-bp tandem duplication (83dup18; g.83_100dupGCTGCTCTGCGCGCTGCTC; c.45_62dupGCTGCTCTGCGCGCTGCTC), 1-bp proximal to the 84dup18 mutation in exon 1 of *TNFRSF11A* [6].

In addition to the FEO cases, a mutation in *TNFRSF11A* (75dup27; g.75_101dupGCGCTGCTGCTCTGCGCGCTGCTC; c.37_63dupGCGCTGCTGCTGCTCTGCGCTGCTC) overlapping the mutations of the FEO patients was identified in affected members of a Japanese early-onset PDB pedigree [4]. Mutations in *TNFRSF11A* have not been identified in the many classic late-onset PDB patients screened [2]. Finally, a fourth mutation in *TNFRSF11A* (85dup15; g.84_98dupTGCTCTGCGCGCTGCTC; c.46_60dupCTGCTCTGCGCGCTGCTC), overlapping with the previous three, has been reported in an Australian family afflicted with expansile skeletal hyperphosphatasia (ESH) [9]. Although ESH was thus genetically allelic to FEO, its phenotypic features were considered distinct. Specifically, ESH was characterized by widespread cortical bone thickening, absence of osteolytic lesions producing cortical thinning in the major long bones, and bouts of hypercalcemia.

Here, we report the existence of the same *TNFRSF11A* 84dup18 mutation (g.84_101dupCTGCTCTGCGCGCTGCTC; c.46_63dupCTGCTCTGCGCGCTGCTC) previously found in four FEO pedigrees in an Iranian FEO family. An associated haplotype is described, which suggests that the origin of the mutation in this family is distinct from that of the same mutation in the Irish, American, and German pedigrees, and that the mutation has at least three different origins.

Materials and methods

Subjects

This study was performed after approval from the Ethics Review Board of the National Institute for Genetic Engineering and Biotechnology (Iran) and with informed con-

Fig. 1. Pedigree of Iranian familial expansile osteolysis (FEO) family

sent of pedigree members in accordance with the Declaration of Helsinki Guidelines. The proband is a 31-year-old woman of an Iranian FEO pedigree that has previously been described, with emphasis on otological features of the patients [10]. Disease in the pedigree, which contains six affected individuals distributed in three generations, shows autosomal dominant inheritance (Fig. 1). Members of the pedigree not affected with FEO were healthy. Family members report no consanguinity.

The phenotype of the proband is more severe than that of other affected members of the pedigree. Some clinical features not presented in the previous report on the pedigree were detected in a thorough examination of the proband at the age of 31 [10]. Whole-body Tc99-MBP (technetium 99-methylene biphosphonate) scan revealed multiple sites of increased activity, particularly in the calvarium, facial bones, clavicles, long bones of the extremities, left fifth metatarsal bone, left foot second phalangeal bone, and medial tarsal joints. Bone densitometry by dual-energy X-ray absorptiometry (DXA) showed low bone mass in the wrist (T -score, -2.6) and lumbar (T -score, -3.4) regions. Bone density of the femoral neck was normal. Radiography showed anterior bowing of the tibia and coarse longitudinal trabeculation in the metaphysis (Fig. 2a). Local lesions in the proximal part of the right tibia and degenerative changes in both knee joints were observed. Hand X-rays revealed diffuse expansion of bones and a cystic pattern of coarse trabeculation (Fig. 2b). Radiographs of the pelvis were normal, but X-rays of the skull indicated increased thickness of the occipital bone (Fig. 2c). Treatment of this patient with alendronate has been initiated.

A recent medical examination on other affected pedigree members was not performed, either because they were not available or they would not consent. Nevertheless, some information is available from previous medical records or conversations. Individuals III:3 and III:6 have more severe phenotypes than the other affected individuals. Phenotypic variations within and between FEO pedigrees have been reported in other studies [2]. Although the clinical features of the Iranian pedigree most closely resemble those of FEO, hand and skull involvement has previously been associated

Fig. 2. Radiologic findings of proband of Iranian FEO pedigree. **a** X-ray shows anterior bowing of both tibia with coarse longitudinal trabeculation in their metaphysis. A lucent defect (2.5 cm × 1.5 cm) in the proximal part of the right tibia and degenerative changes in both knee joints are seen. **b** Diffuse expansion of hand bones with coarse trabeculation are visible, and cystic changes without subperiosteal resorption are noted. **c** Skull radiograph shows increased thickness of occipital part of cranial bones

Table 1. Phenotypic features of familial expansile osteolysis (FEO) patients

Nationality	Spanish [5]	Irish [2,4]	American [2,4]	German [2,4]	Sporadic [6]	American [6]	Iranian (present study)
Deafness in childhood	Yes	Yes	Yes	NR	Yes	Yes	Yes
Tooth loss	Yes (in childhood)	Yes (in childhood)	Yes (in childhood)	NR	Yes (late twenties)	Yes (during twenties)	Yes
Hand involvement	No	No	No	Yes	No	No	Yes
Pelvic involvement	No	No	No	NR	No	No	No
Skull involvement	No	No	No	NR	No	No	Yes
Pathological fracture	No	NR	NR	NR	Yes	NR	Yes
Osteolytic lesions	Only in 2 of 14 affected	Yes	Yes	Yes	Yes	NR	Yes
Elevated SAP	NR	Yes	Yes	NR	Yes	Yes	Yes
Elevated UHP	NR	NR	NR	NR	NR	Yes	NR

NR, not reported; SAP, serum alkaline phosphatase; UHP, urinary hydroxyproline

with ESH and early-onset PDB and generally not with FEO, emphasizing the merging phenotypic features of these diseases [11].

Table 1 compares the reported phenotypic features of genetically analyzed FEO patients thus far reported. Even childhood deafness, the earliest sign of FEO in most patients, does not manifest in all affected individuals (e.g., III:5 in Iranian pedigree showed no sign of deafness at age 28). Although early-onset deafness was not observed in most affected members of the early-onset PDB pedigree carrying a mutation in *TNFRSF11A*, it was reported in one of the patients [11]. Similarly, tooth loss is generally in childhood in FEO and later in ESH and PDB, but the reverse pattern has also been reported in some cases [6,11].

DNA analysis

DNA was prepared from the leukocytes of the peripheral blood of individuals II:4, III:2, III:3, III:6, and IV:1. Exon 1 of *TNFRSF11A* was amplified by the polymerase chain reaction (PCR) using forward primer 5'-TGGGGTGCG GCAAGGCAGGAG-3' and reverse primer 5'-AAGGC GGAGGAGCCAGGATGC-3'. Exon 1 amplicons were electrophoresed on agarose gels. The remaining 9 exons of *TNFRSF11A* were PCR amplified only from DNA of the proband. (Primer sequences are available upon request.) All DNA bands observed upon electrophoresis of exon 1 PCR products of affected individuals were eluted and sequenced using the ABI Big Dye terminator chemistry. In all, 6217 nucleotides in the DNA of the proband were

sequenced, including 303 nucleotides upstream of the transcriptional initiation site, the entire 5'-noncoding and 252 nucleotides of the 3'-nontranslated region. Sequence variations were determined by comparison with the NCBI genomic DNA reference sequence (NT_025028), cDNA reference sequence (NM_003839), and protein reference sequence (NP_003830), using Sequencher software (Gene Codes Corporation, Ann Arbor, MI, USA). Effects of sequence changes on splicing were predicted by comparison with known canonical splice site motifs (http://www.fruitfly.org/seq_tools/splice.html). For determination of the phase of two variations found in the DNA of the proband by sequencing, linkage analysis was done by RFLP using *Aci* I (Cinnagen, Tehran, Iran).

Results

The electrophoresis pattern of the PCR product of exon 1 of the *TNFRSF11A* gene in DNA of affected family members was consistent with there being an insertion in one allele of each of the individuals (Fig. 3). Sequencing showed that exon 1 of the proband and afflicted individuals II:4 and III:6 carried an 18-bp tandem duplication, g.84_101dupCTGCTCTGCGCGCTGCTC (c.46_63dupCTGCTCTGCGCGCTGCTC) in the heterozygous state (Fig. 4); this is the same duplication as reported in four previously studied FEO pedigrees [4,5]. Both alleles of the unaffected 28-year-old sister (III:2) of the proband were wild type, as were the alleles of the 6-year-old offspring of the proband.

In addition to the disease-causing mutation, the DNA sequence of the proband showed eight other sequence variations as compared to the *TNFRSF11A* genomic sequence NT_025028. One variation caused an amino acid alteration, another resulted in a synonymous codon change, and six were intronic. The variations are listed in Table 2. None of the variations were predicted to affect splicing. The variations g.34694C>T (c.575C>T; p.A192V), IVS5-17C>T, and IVS6-151G>A were present in the heterozygous state, and the others were homozygous. Linkage analysis by RFLP on

Fig. 4. Sequence chromatograms of exon 1 amplicon of proband. **A** DNA of wild-type allele; **B** DNA of mutated allele; **C** DNA of heteroduplex band. Relevant sequences are underlined, and coded amino acids are indicated above chromatograms **A** and **B**

the DNAs of individuals II:4, III:2, III:3, and III:6 showed g.34694C (c.575C; p.192A) was linked to the mutation-carrying allele of *TNFRSF11A*. The same analysis proved to be uninformative for the variation IVS5-17C>T (not shown). Phase analysis was not done for IVS6-151G>A.

Discussion

A fifth FEO pedigree carrying the disease causing g.84_101dupCTGCTCTGCGCGTCTC duplication (84dup18) in *TNFRSF11A* is reported here. The 83dup18 mutation found in two other unrelated FEO cases has the same effect as 84dup18 at the protein level; both result in the same six amino acid insertion in the signal peptide of RANK [6]. The finding of either of two mutations that result in an identical protein alteration in all the FEO cases suggests this particular alteration may uniquely result in the FEO phenotype. Other mutations in the gene may cause

Table 2. Variations in *TNFRSF11A* in FEO pedigrees

SNP			rs ^{#c}	Nucleotide in reference sequence	Linkage to mutated allele in FEO pedigrees			
g. position ^a (NT_025028)	c. position ^{a,b} (NM_003839)	p. position (NP_003830)			Irish	US	German	Iranian
g.118	IVS1+5			G	A	G	G	T
g.34624	IVS5-17		6567272	C	C	?	?	C/T ^d
g.34694	c.575	A192	1805034	C	C	?	?	C ^c
g.34814	IVS6+79		9653064	G	G	?	?	A
g.36215	IVS6-151		8099222	G	?	G	?	G/A ^d
g.36670	IVS7+192			T	?	?	?	C
g.36691	IVS7+213		7239667	G	?	?	?	C
g.43536	c.933	T311	8092336	A	G	G	?	G

^a Positions where nucleotide definitely differ between two or more pedigrees are shown in bold

^b "A" of AUG initiation codon is considered +1

^c Reference numbers (rs#) of variations registered in build 125 of NCBI SNP database (<http://www.ncbi.nlm.nih.gov>)

^d Phase not established

^e Nucleotide on allele not carrying the duplication mutation was T

diseases with related and overlapping yet distinct phenotypic features, such as early-onset PDB and ESH [4,9]. Alternatively, it is possible that environmental factors and the genetic background of the patients significantly contribute to the phenotypic variations of patients carrying the different mutations in *TNFRSF11A* [12]. It will be interesting to learn the genetic cause of FEO in a recently reported FEO pedigree from the Czech Republic [13].

The haplotype associated with the mutated *TNFRSF11A* allele in the Iranian pedigree, defined by six intragenic single nucleotide variations, five of which were present in the homozygous state, can unambiguously be described as T_{g.118} C_{g.34694} A_{g.34814} C_{g.36670} C_{g.36691} G_{g.43536} (c.T_{IVS1+5} C_{c.575} A_{IVS6+79} C_{IVS7+192} C_{IVS7+213} G_{c.933}). There has previously been only one report on single nucleotide variations associated with the mutated *TNFRSF11A* allele in FEO pedigrees [4]. In Table 2, nucleotides at positions corresponding to some of the variant positions found in the Iranian pedigree are shown in three other FEO pedigrees carrying the same 84dup18 mutation. The pedigrees do not share the same allele at positions IVS1+5 and IVS6+79. Most interestingly, position IVS1+5, close to the 5'-splice site of intron 1, was A in the Irish pedigree, G in the American and German pedigrees, and T in the Iranian pedigree. This finding implies that the common mutation in the Irish, American, and Iranian pedigrees each arose independently. The mutated allele of the American and German pedigrees may have a common origin based on this analysis. G is found at position 5 of the 5'-splice site in more than 75% of human introns, and A and T are both relatively rare alleles at that position [14]. The independent origin of the mutation in the Irish and Iranian pedigrees is confirmed by the difference of sequence at position IVS6+79. Knowledge of SNP nucleotides in *TNFRSF11A* and the haplotypes they define will be useful for determining common or independent origins of FEO mutations to be identified in other pedigrees in the future.

The 84dup18 duplication observed in the FEO pedigrees and the 27bp duplication in the early-onset PDB pedigree were previously suggested to have arisen by reverse slippage during DNA replication [4]. A model that suggests the role of a stable secondary structure of the DNA in facilitat-

ing generation of the tandem duplications has also been presented [6]. The proposed independent origins of the identical duplication in at least three different FEO pedigrees suggest that the duplication occurs at a mutation hot-spot and is consistent with these models. The repetitive and G/C-rich nature of the sequences surrounding the close and partly overlapping duplications of the FEO, early-onset PDB, and ESH duplications are likely to promote mutation events [15]. The preponderance of CTG repeats may be particularly important in this regard [16].

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