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Mutation Screening of the *TNFRSF11A* Gene Encoding Receptor Activator of NFκB (RANK) in Familial and Sporadic Paget's Disease of Bone and Osteosarcoma

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Abstract. Paget's disease of bone (PDB) is a common disorder characterized by focal areas of increased and disorganized osteoclastic bone resorption, leading to bone pain, deformity, pathological fracture, and an increased risk of osteosarcoma. Genetic factors play an important role in the pathogenesis of Paget's disease. In some families, the disease has been found to be linked to a susceptibility locus on chromosome 18q21-22, which also contains the gene responsible for familial expansile osteolysis (FEO)—a rare bone dysplasia with many similarities to Paget's disease. Insertion mutations of the *TNFRSF11A* gene encoding Receptor Activator of NFκB (RANK) have recently been found to be responsible for FEO and rare cases of early onset familial Paget's disease. Loss of heterozygosity (LOH) affecting the PDB/FEO critical region has also been described in osteosarcomas suggesting that *TNFRSF11A* might also be involved in the development of osteosarcoma. In order to investigate the possible role of *TNFRSF11A* in the pathogenesis of Paget's disease and osteosarcoma, we conducted mutation screening of the *TNFRSF11A* gene in patients with familial and sporadic Paget's disease as well as DNA extracted from Pagetic bone lesions, an osteosarcoma arising in Pagetic bone and six osteosarcoma cell lines. No specific abnormalities of the *TNFRSF11A* gene were identified in a Pagetic osteosarcoma, the osteosarcoma cell lines, DNA extracted from Pagetic bone lesions, or DNA extracted from peripheral blood in patients with familial or sporadic Paget's disease including several individuals with early onset Paget's disease. These data indicate that *TNFRSF11A* mutations contribute neither to the vast majority of cases of sporadic or familial PDB, nor to the development of osteosarcoma.

Key words: Paget's disease — Osteosarcoma — DNA — genetic — *TNFRSF11A*

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Paget's disease of bone (PDB; MIM 167250) is among the most common diseases of adults, affecting about 3% of individuals over 55 in Caucasian populations [1]. Paget's disease is characterized by focal areas of increased and disorganized osteoclastic bone resorption coupled with new bone formation, which leads to bone pain, deformity, osteoarthritis, and an increased risk of pathological fracture [12]. Moreover, affected individuals suffer a significantly increased risk of developing osteosarcoma. Pagetic individuals comprise more than half of all patients over the age of 60 who develop osteosarcoma [8].

The cause of PDB remains unclear [10], but accumulating evidence indicates that there is a strong genetic component. Familial clustering is common, and between 15 and 40% of Pagetic patients have an affected first-degree relative with the disease [5–7]. Several families have also been described where Paget's disease is inherited in an autosomal dominant manner [5, 13–15], as is the rare bone dysplasia Familial Expansile Osteolysis (FEO; OMIM 174810), which shares many clinical features with PDB [16, 17]. Hughes et al. mapped the gene responsible for FEO to the interval D18S64-D18S42 on chromosome 18q21-22 in 1994 [17] and subsequently, several Paget's kindreds were found to exhibit linkage to this interval [4, 13]. In addition, loss of heterozygosity (LOH) of 18q in osteosarcomas was mapped to within the FEO/PDB critical region [18], suggesting that the same gene might be involved in the pathogenesis of FEO, PDB, and in the development of osteosarcoma. Recent studies by Hughes et al. [19] mapped the *TNFRSF11A* gene which encodes the RANK protein to within the FEO/PDB critical region and identified a six amino acid insertion mutation affecting the signal peptide region of the protein, which segregated with the disease in affected individuals from three families with FEO, and a nine amino acid insertion mutation, which segregated with the disease in a family with a phenotype of severe, early

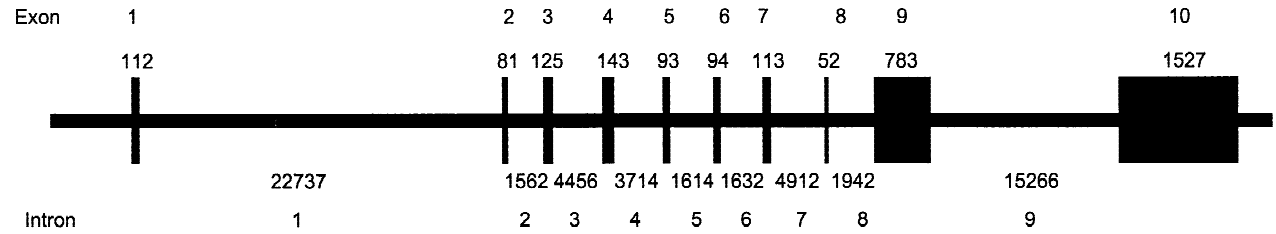

Fig. 1. Details of BAC contig containing the TNFRSF11A gene.

onset Paget's disease. In this study, we further investigated the role of the *TNFRSF11A* gene in the pathogenesis of Paget's disease and osteosarcoma by mutation screening of the gene in individuals with sporadic and familial Paget's disease; in somatic DNA extracted from Pagetic bone lesions; in osteosarcoma arising in Pagetic bone; and in seven osteosarcoma cell lines.

Materials and Methods

Patient Samples

Blood samples were collected from six patients with early onset PDB (age of onset below 40 years) and affected individuals from 64 families with autosomal dominant inheritance of PDB, 3 of whom had shown weak evidence of linkage to the PDB/FEO critical region on chromosome 18q21-22. Details of these families have been described elsewhere [14]. Additional studies comparing allele frequencies of polymorphisms identified in the *TNFRSF11A* gene were performed in unrelated patients with sporadic Paget's disease and aged-matched controls from the UK. All patients gave informed consent to participating in the studies, which were approved by the Johns Hopkins University Joint Committee on Clinical Investigation and by the Joint Ethical Committee of Aberdeen Royal Hospitals NHS Trust. Analysis of the *TNFRSF11A* gene was also performed in DNA extracted from an osteosarcoma arising in Pagetic bone, and in the osteosarcoma cell lines SJSA-1, U-2OS, SK-ES-1, SAOS2, CRL-8303, and CRL-1544, which were obtained from the ATCC.

Molecular Biology

RNA was isolated from cell lines using the RNAgents kit (Promega, Madison, WI) according to the manufacturer's instructions (except for the exclusion of sodium acetate in the lysis buffer during DNA extraction) and was extracted from bone samples using RNAzol (Biogenesis, Dorset UK) as previously described [20]. cDNA was prepared by using the Superscript kit (BRL, UK) according to the manufacturer's instructions. DNA was isolated from peripheral blood samples using the Nucleon Kit (Scotlab, UK) and from samples of bone tissue by homogenization in 3M guanidinium isothiocyanate/10% sarcosyl, followed by phenol/chloroform extraction and ethanol precipitation. The *TNFRSF11A* genomic sequence was determined by sequencing a BAC clone (390j15) which had been identified as part of a BAC contig encompassing the PDB/FEO critical region (Fig. 1). The BAC contig was assembled by identifying STS markers which had been mapped to the critical region using public databases including the NCBI GeneMap'98 (http://www.ncbi.nlm.nih.gov/genemap98/) and the SHGC G3 radiation hybrid map (http://www-shgc.stanford.edu/Mapping/rh/). BAC clones containing these markers were identified by PCR-based screening of the CITB human BAC library (Research Genetics, Huntsville, AL, USA) as previously described [21]. Additional STS markers were identified from distal BAC end sequence data and were used to re-screen the BAC library, thereby extending the contig and ultimately establishing overlaps between BAC islands. The sequence of the region was determined by automated sequencing of subclones derived from the minimal tiling path BACs. The intron-exon structure of the *TNFRSF11A* gene was defined by comparing the BAC sequence data with the cDNA sequence of *TNFRSF11A* [3] (GenBank Accession: AF019253) and primers were designed to amplify the proximal promoter region (from nucleotide −152, where + 1 is the mRNA start site) and each of the 10 *TNFRSF11A* exons and associated 5′ and 3′ splice junctions (Table 1). PCR products were gel-purified and evaluated for sequence alterations using a combination of automated and manual sequencing as described [22].

Results

The *TNFRSF11A* gene was found to span 61 Kb of genomic DNA and to comprise 10 exons of 112, 81, 125, 143, 93, 94, 113, 52, 783, and 1527 bp in length, intervened by nine introns of 22737, 1562, 4456, 3714, 1614, 1632, 4912, 1942, and 15,266 bp in length, respectively (Fig. 2). This sequence has been deposited in GenBank (accession number AF298900). Each of the 10 exons encoding RANK, including its associated splice junctions, and the proximal promoter region were amplified by PCR of genomic DNA from patient samples and evaluated for the presence of mutations. As a result of this process we identified several polymorphisms of *TNFRSF11A*, most of which were described in a previous study [19]. Three novel polymorphisms affecting the coding region of RANK were found, but one of these (T311T; ACG/ACA) was only detected in the CRL-8303 osteosarcoma cell line. The other polymorphisms were found to be present in normal controls as well as Pagetic patients at similar allele frequencies (Table 2). We found no other specific mutations in six osteosarcoma cell lines originating in the absence of PDB, nor in a primary osteosarcoma derived from Pagetic bone. We similarly failed to detect disease-specific polymorphisms or mutations of *TNFRSF11A* in constitutional DNA from six young Paget's patients, each under 40 years of age at disease onset, or in constitutional DNA from three PDB kindreds where the disease had segregated within the family with a common 18q haplotype. Affected individuals from one of these families were found to have a variant in the splice donor site of intron 1 (IVS1 + 5G → A) that segre-

Table 1. Oligonucleotides used for PCR amplification of *TNFRSF11A*

Exon	5′	3′
1	CGCAAACCAGGGGAGCTTGG	GGGGCTCCCGAGGAGGCGTCG
2	TTATCCAGAAAGAGCTGTGTG	AATAATGAAAGCCTCACCCAC
3	GGGTTATTTCAGCCAGCTGCG	CACTGACACACTGACAGGACG
4	TGGATAGCGCAGTCGTGGGCG	TGCATGGCTCAGCAAGAGCCG
5	CCTGGATGATCTCTAAGTGAC	AGCCTAACCCAACAGAATCCG
6	AAAACCAAAGCACTGAACCACC	CCCTGGGGCACATCTATCAAC
7	ATCTTTACTACCATATTTCTC	CTCAACTTCCAAAGCATTTACC
8	AACTTGAAGTCCTTATCCTTGC	GGTGCTCAATATGTGACTAGG
9	ACTTGTCCTAATAACTCTAGG	CAAACCCTGTACCAAGAACTG
10 CDS	GGAACCTTCCTCTCGGCAGAC	GACATCTGCCCCAGTGCGGCG
10 UTR	GGCCACCCAGGGATCGATCG	AGGCTAACCCACAGATGCACG

CDS–coding sequence; UTR–untranslated region

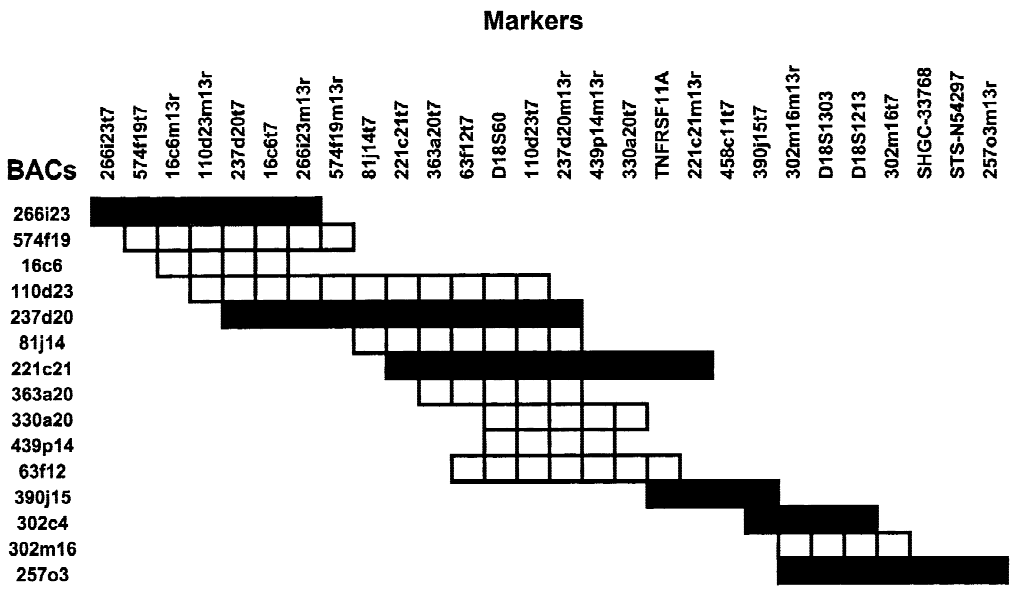

Fig. 2. Intron exon structure of the *TNFRSF11A* gene. The numbers and sizes of exons (vertical bars) and introns (horizontal line) are indicated in bp (intron sizes are not drawn to scale).

Table 2. Coding region polymorphisms of *TNFRSF11A*

			Allele Frequency		Individuals Genotyped	
Exon	Codon	Polymorphism	Paget's	Controls	Paget's/Controls	*P*-value
4	H141Y	CAC/TAC	22.0%	19.7%	41/33	0.73
6	A192V	GCG/GTG	47.9%	48.5%	47/34	0.93
9	G429G	GGC/GGA	3.3%	4.7%	30/21	0.79

P-values refer to the differences in allele frequency between Paget's patients and controls

gated with disease. The IVS1 + 5G → A variant was also found in DNA derived from Pagetic bone in 2/6 cases studied, but analysis of leukocyte DNA from one of these individuals revealed the presence of the IVS1 + 5A variant, precluding somatic acquisition. To evaluate whether the variant altered the transcription product of *TNFRSF11A*, gel electrophoresis and manual sequencing of RT-PCR products from lymphoid cells of a PDB patient with the IVS1 + 5A variant were carried out and a coding polymorphism in exon 4 (Table 2) was used to assess the expression level of each allele. This analysis revealed no difference in the cDNA sequence or in the expression level of the IVS1 + 5A allele (data not shown). We finally screened exon 1 of the *TNFRSF11A* gene for insertion mutations similar to those

described previously [19] in 64 additional families with autosomal dominant familial Paget's disease [14] but no abnormalities were detected.

Discussion

Recent work has shown that mutations in the *TNFRSF11A* gene cause the Paget's disease-like bone dysplasia FEO and some cases of severe early onset Paget's disease [19]. The RANK molecule and its ligand RANKL were originally identified as modulators of T-cell growth and dendritic cell function by Anderson et al. in 1997 [3]. Subsequent work showed that the RANK-RANKL signaling pathway was essential for osteoclastogenesis as well as lymph node organogenesis [23–29]. The mutation responsible for FEO in the families previously described [19] was a heterozygous 18bp insertion affecting exon 1 of the *TNFRSF11A* gene. This causes an in-frame insertion duplication of 6 amino acids, resulting in failure of signal peptide cleavage and activation of NFκB signaling. In the same study, a 27bp insertion mutation affecting the same region of the molecule was found in one other family where the clinical picture was consistent with early-onset Paget's disease. As in the case of 18bp insertion, this mutation impaired signal peptide cleavage and caused NFκB activation. Hughes and colleagues failed to detect similar mutations in 90 patients with sporadic PDB, in 3 families with weak evidence of linkage to 18q, or in cDNA prepared from Pagetic bone, suggesting that *TNFRSF11A* mutations are infrequent in Paget's disease.

In this study, we conducted extensive screening of the *TNFRSF11A* gene for mutations in patients with early onset sporadic Paget's disease, subjects from families with Paget's disease where affected individuals shared a common haplotype on 18q21-22, DNA extracted from Pagetic bone lesions, osteosarcoma cell lines, as well as DNA extracted from a Pagetic osteosarcoma. No disease-specific polymorphisms or mutations were identified as the result of these studies, which supports the findings of previous work suggesting that mutations of the *TNFRSF11A* gene are rare in sporadic Paget's disease. We also screened for the presence of insertion mutations in exon 1 of the gene in affected individuals from 64 families with autosomal dominant Paget's disease. Whereas previous studies in this cohort of families did not support linkage to 18q21-22 [14] the individual families were too small to definitively exclude linkage. The data reported here now indicate that insertion mutations of RANK can definitely be excluded as a cause of the disease in these families. In addition to the polymorphic sequence variants reported previously [19] we detected 2 novel polymorphisms affecting the coding region of RANK in leukocyte DNA. One of these was a conservative change however and all polymorphisms studied were found at similar allele frequencies in Pagetic and control subjects, suggesting that they were unrelated to the pathogenesis of Paget's disease. These data are in broad agreement with the findings previously reported by Hughes, who found no association between intronic polymorphisms of RANK and sporadic Paget's disease in a population of Pagetic patients from Northern Ireland [19]. We also investigated the possible functional consequences of the IVS1 + 5A splice site variant found by Hughes [19] to segregate with FEO in a large family from Northern Ireland. Whereas IVS1 + 5A mutations are associated with several hereditary diseases and represent the second most common target for splice site mutations recorded in the literature [2], we found no evidence to suggest that the IVS1 + 5A variant altered splicing or allele-specific transcription. This suggests that IVS1 + 5A variant in *TNFRSF11A* is a benign sequence variant. We also screened DNA extracted from Pagetic bone for the presence of mutations, but none were found, excluding the possibility that the disease may arise as the result of somatic mutations in *TNFRSF11A*. Finally, we failed to detect specific mutations of *TNFRSF11A* in osteosarcoma cell lines and DNA extracted from a Pagetic osteosarcoma, indicating that somatic mutations of *TNFRSF11A* are unlikely to explain the development of osteosarcoma. Only one clinical osteosarcoma was studied however and further work will clearly be required to replicate these observations. It is important to emphasise that we cannot exclude the possibility that allele loss affecting *TNFRSF11A* and/or other genes within this region might contribute to the pathogenesis of Pagetic osteosarcoma as suggested by the work of Nellissery [18], but further work will be required to investigate this hypothesis.

In conclusion, these data when taken together with those previously reported [19] indicate that *TNFRSF11A* mutations do not contribute to the vast majority of cases of sporadic or familial PDB, nor to the development of osteosarcoma.

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