

ARTICLE

Linkage to chromosome 11p12 in two Maltese families with a highly penetrant form of osteoporosis

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Osteoporosis is a metabolic bone disease with a strong genetic component. Family-based linkage studies were performed by a number of investigators to try to identify loci that might contain genes responsible for an increased susceptibility to osteoporosis. A whole-genome linkage scan using 400 microsatellite markers was performed in 27 members from two Maltese families with a highly penetrant form of osteoporosis. The phenotype was defined by lumbar and femoral z-scores calculated after measurement of bone mineral density by DEXA. Both males and females were among the affected individuals. Multipoint parametric and non-parametric linkage analyses were performed by EasyLinkage v4.01 using GENEHUNTER v2.1, assuming dominant and recessive modes of inheritance with variable penetrance. Evidence of linkage was observed to a marker at 11p12 where a non-parametric LOD score of 5.77 ($P=0.0006$) was obtained. A maximum heterogeneity LOD score of 2.55 for this region was obtained for the dominant mode of inheritance with 90% penetrance and a phenocopy rate of 1%. Following fine mapping, the critical interval was narrowed to a region that is 52.94 cM from 11p-telomere. In this region, the gene for tumour necrosis factor receptor-associated factor 6 (*TRAF6*) is located approximately 1 cM away from the indicated marker. Sequencing of the promoter region and exons of the *TRAF6* gene revealed three sequence variants, one of which was found in three affected members within one family. *European Journal of Human Genetics* (2007) 15, 800–809; doi:10.1038/sj.ejhg.5201814; published online 21 March 2007

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Introduction

Osteoporosis (MIM 166710) is a polygenic disease where multiple gene variants, each having a small effect, contribute to the individual's increased susceptibility to the disease, although a major gene might also be involved.¹ The search for genes responsible for complex diseases, such as osteoporosis, has presented a great challenge for geneticists during the last decade. Different approaches, such as case–control association and family-based linkage,

have been used to identify any association between phenotype and gene variants.²

Family-based linkage studies were performed by a number of investigators to try to identify loci that might contain genes responsible for an increased susceptibility to osteoporosis. A number of chromosomal loci have been identified and confirmed to quantitative trait loci (QTL), such as bone mineral density (BMD), by whole-genome scans and by scanning candidate regions. Duncan *et al*³ observed suggestive linkage to the parathyroid hormone receptor type 1 (*PTHRI*) on chromosome 3p21 in a study carried out on a large group of British families. Moderate evidence of linkage was also reported at loci where other candidate genes are found including the *COL1A1*, *COL2A1*/*VDR*, *IL-6* and oestrogen receptor 1. Wilson *et al*⁴ observed

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linkage with BMD at the lumbar spine (LS-BMD) with loci 3p21 and 1p36 as well as with other chromosomal loci including 11p, 2q and 19q, thus confirming the results obtained by Duncan *et al*³ and Devoto *et al*,⁵ where the latter associated locus 1p36 with low-hip BMD, after performing a non-parametric linkage analysis in seven extended families. Devoto *et al*⁶ confirmed these results after performing a higher resolution scan in an extended sample of 40 families coming from five populations of mixed ethnicity (Greeks, Italians, French, Jewish and Middle Eastern).

Another interesting locus is 11q12–13, which has been associated with peak bone mass⁷ as well as with other phenotypes including autosomal recessive osteopetrosis,⁸ high bone mass phenotype⁹ and osteoporosis pseudoglioma syndrome.¹⁰ A number of loci were confirmed by linkage to a QTL, such as BMD at different anatomical sites,^{11,12} variation in femoral structure¹³ and peak bone mass reached early in life.¹⁴

Linkage and association were confirmed to chromosome 20p12 in a study carried out on the Icelandic population.¹⁵ Discrete phenotypes were defined by using z-scores corrected for age and combined with fractures of different degrees of severity. Linkage was observed at chromosome 20p12. Linkage disequilibrium mapping using a number of single-nucleotide polymorphisms (SNPs) at this region indicated a missense polymorphism and two SNP haplotypes within the bone morphogenic protein-2 (*BMP2*)

gene, which were associated with osteoporosis in both the Icelandic and Danish populations.¹⁵ This region was later confirmed in a study of European Caucasians where evidence of linkage to regions 11q23 and Xq27 was also observed.¹⁶

In this study, a whole-genome-wide scan was performed in two extended Maltese families with a highly penetrant form of osteoporosis, for possible identification of loci that might be responsible for osteoporosis in the Maltese population.

Materials and methods

Subjects

Two extended families with a high incidence of osteoporosis were included in this study (Figures 1 and 2). These families were recruited from the Department of Obstetrics and Gynaecology, St Luke's Hospital, Malta and selected from probands that already participated in previous association studies.^{17,18} These two probands were observed to be severely osteoporotic according to WHO criteria (*t*-score < -2.50), where both probands had *t*-scores of -3.50, were relatively young in age when diagnosed (50 and 55 years, respectively) and have a known family history of osteoporosis. A total of 27 family members were recruited, nine of whom came from pedigree 1 and 18 from pedigree 2 (Table 1). All participants were asked to sign an informed consent form and answered a detailed questionnaire

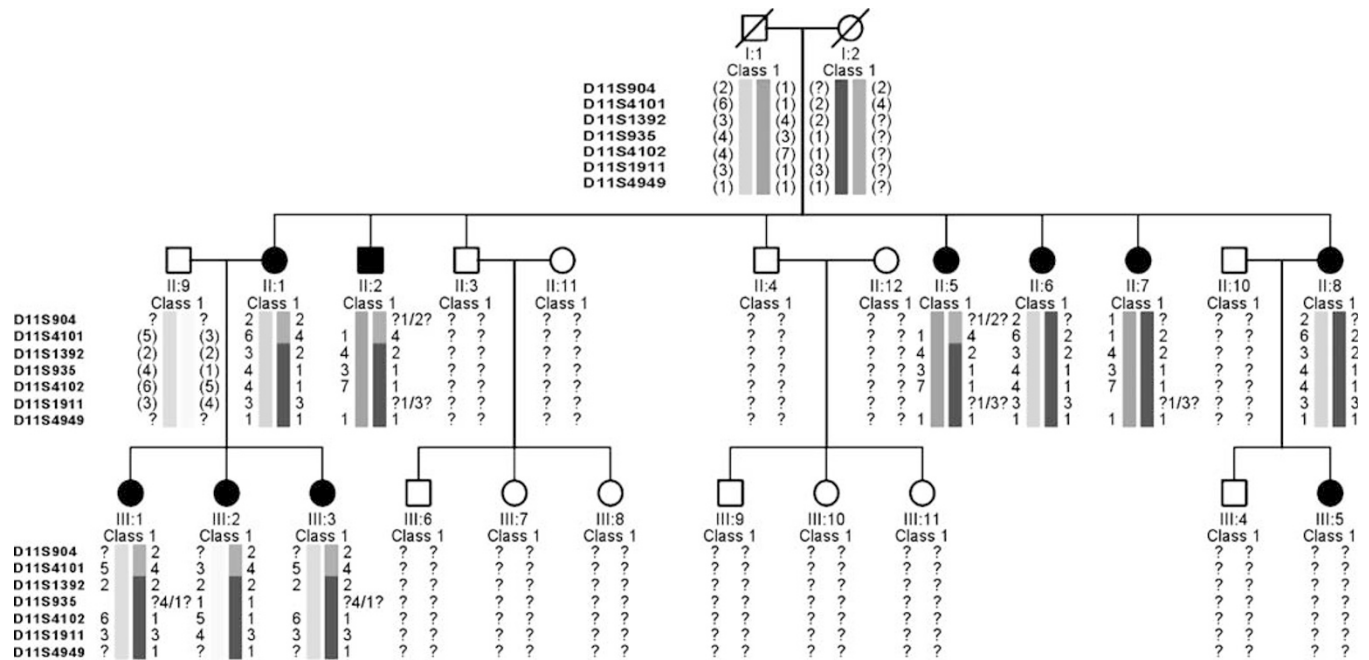


Figure 1 Pedigree 1 showing inheritance of part of chromosome 11. Affected individuals (black shaded) in generation II where defined according to the WHO criteria (*t*-score < -2.5). In generation III, affected status was defined by z-score < -1.0. Individuals II:3 and II:4 were not recruited, therefore affected status is unknown. DNA was not obtained from individual III:5. Inferred genotypes were generated by GENEHUNTER and are shown in parentheses.

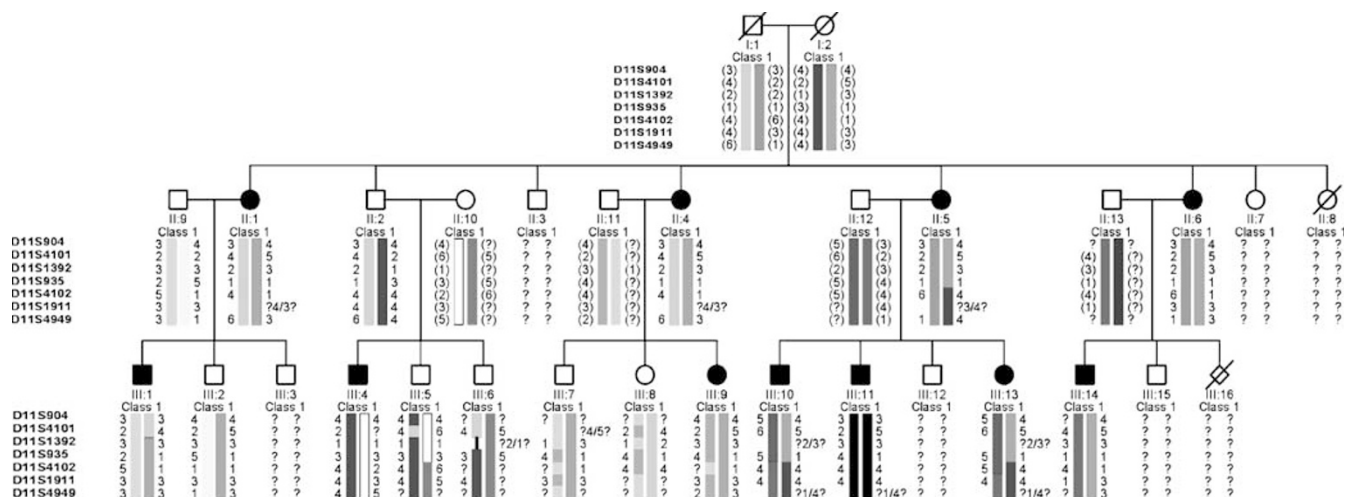


Figure 2 This figure shows pedigree 2 and inheritance of part of chromosome 11. Affected individuals (black shaded) in generation II where defined according to the WHO criteria (t -score < -2.5). In generation III, affected status was defined by z -score < -1.0 . Individuals II:3, II:7, III:3, III:12 and III:15 were not recruited, therefore affected status is unknown. Inferred genotypes were generated by GENEHUNTER and are shown in parentheses.

Table 1 Clinical characteristics of family members

Pedigree 1	age	BMI	LS t-score (z-score)	FN t-score (z-score)	Fracture
II : 1 ^a	61	29.5	-3.50 (-1.44)	-1.51 (-0.18)	Nil
II : 2	74	23.1	-2.29 (-1.54)	-3.29 (-1.85)	Nil
II : 5	62	28.8	-3.28 (-1.20)	-0.84 (0.56)	Nil
II : 6	72	34.2	-4.16 (-1.91)	-2.42 (-0.79)	Vertebral #
II : 7	70	26.2	-3.90 (-1.65)	-2.12 (-0.53)	Nil
II : 8	68	24.1	-5.68 (-3.09)	-2.25 (-0.70)	Nil
III : 1	33	22.5	-1.93 (-1.83)	-1.37 (-1.42)	Scaphoid #
III : 2	35	23.9	-2.49 (-2.29)	-1.89 (-1.86)	Nil
III : 3	38	23.0	-2.14 (-1.86)	-1.24 (-1.10)	Nil
Pedigree 2					
II : 1 ^b	55	26.5	-3.47 (-1.68)	-1.57 (-0.56)	Nil
II : 2	66	27.0	-1.19 (0.57)	-0.19 (0.62)	Clavicle #
II : 4	61	28.8	-2.66 (-0.78)	-1.70 (-0.37)	Nil
II : 5	70	29.1	-2.88 (-0.78)	-2.24 (-0.65)	Nil
II : 6	52	21.2	-3.75 (-2.18)	-2.72 (-1.86)	Calcaneus #
III : 1	29	25.3	-2.00 (-2.02)	-1.22 (-1.09)	Nil
III : 2	23	19.7	-1.58 (-0.87)	-1.10 (-0.36)	Nil
III : 4	41	30.1	-1.67 (-1.42)	-0.86 (-0.45)	Wrist #
III : 5	38	23.5	-0.13 (-0.03)	-0.57 (-0.37)	Nil
III : 6	33	24.1	-0.62 (-0.60)	-0.27 (0.25)	Nil
III : 7	33	31.8	-0.42 (-0.36)	-0.24 (0.04)	Nil
III : 8	38	30.9	2.64 (2.42)	1.41 (1.56)	Nil
III : 9	42	25.4	-2.10 (-1.62)	-1.77 (-1.40)	Nil
III : 10	37	28.1	-2.32 (-2.19)	-1.10 (-0.76)	Nil
III : 11	42	25.3	-1.39 (-1.13)	-2.08 (-1.66)	Nil
III : 13	39	24.5	-1.46 (-1.19)	-0.56 (-0.33)	Nil
III : 14	25	21.1	-1.28 (-1.36)	-1.45 (-1.43)	Nil

^aIndividual II:1 Family 1 age of diagnosis was 55 years.

^bIndividual II:1 Family 2 age of diagnosis was 50 years.

Individuals are numbered as shown in Figures 1 and 2.

concerning medical history, medication, lifestyle and dietary habits as well as detailed information about family history of bone disease, osteoporosis and fracture. This study was approved by the Research Ethics Committee of the Faculty of Medicine and Surgery, University of Malta.

BMD at the LS and femoral neck (FN) of all participants was measured by DEXA using a Norland 385 (Norland, Medical Systems Inc.) at the Bone Density Unit, St Luke's Hospital, Malta. Peripheral blood was withdrawn from all participants for DNA analysis and biochemical tests in

order to exclude secondary osteoporosis. Biochemical tests performed included serum creatinine, urea, random blood glucose, calcium (total and ionised), magnesium, phosphate, total cholesterol, total bilirubin, alkaline phosphatase, γ -glutamyl transferase, alanine transaminase together with a complete blood count. All biochemical tests were performed using a Hitachi 917 analyser (Boehringer Mannheim, Germany) and a Sysmex K4500 haematology analyser was used for the complete blood count. None of the family members were observed to have medical conditions or were on medications known to affect bone metabolism.

Phenotype definition

Five phenotype scenarios were initially analysed in this study. In scenario I, affected individuals for a low BMD phenotype (for chronological age) were defined by age- and sex-adjusted z-scores at LS and/or FN of less than -1.0 . A more severe phenotype was defined by z-scores of less than -1.5 , in scenario II. These z-scores were calculated by using reference data of BMD measurements obtained for sex- and age-matched normal individuals of the same ethnicity (Caucasian).

The phenotype was also defined using the guidelines suggested by the International Society of Clinical Densitometry,¹⁹ where osteoporosis for postmenopausal women and men over 50 years of age was defined using a lumbar and/or femoral *t*-score of less than -2.5 (as recommended by the WHO). On the other hand, z-scores were used for younger individuals where a z-score of less than -1.0 was used as the threshold for an affected status and that of less than -2.0 for a more severe phenotype, for scenarios III and IV, respectively.

For scenario V, analysis was performed, by assuming as affected only those individuals having femoral z-scores of less than -1.0 .

The same five scenarios were further analysed by assuming that all unaffected individuals had an unknown phenotype. This assumption takes into consideration the possibility that any apparently clinically unaffected individuals might be actually affected, thus reducing the chance of obtaining false negative results.

DNA analysis

DNA was extracted and purified from peripheral blood leucocytes by a salting out procedure, adapted from Miller *et al.*²⁰ Four hundred short tandem repeats (STRs) spread across the 22 autosomes and x-chromosome with an average spacing of 8.63 cM and heterozygosity of 0.77 were selected for the initial scan. Genotyping of microsatellite markers was performed by polymerase chain reaction (PCR), and fragment analysis of amplified products was performed using a 3730xl ABI genetic analyzer (PE Applied Biosystems, Foster City, CA). The average performance of the markers for all samples was of 96.96%. Genotyping was

performed commercially at the McGill University and Genome Quebec Innovation Centre, Quebec, Canada.

Linkage analysis

Multipoint parametric and non-parametric linkage analyses were performed by EasyLinkage v4.01 (http://www.uni-wuerzburg.de/nephrologie/molecular_genetics/molecular_genetics.htm),²¹ using the software GENEHUNTER v2.1 and GENEHUNTER v1.2 to calculate Zlr scores using Kong and Cox model.²² The Zlr algorithm addresses the problem with previous versions of GENEHUNTER where non-parametric LOD (NPL) scores are found to be too conservative when inheritance data are not complete.

Parametric analysis was carried out using variable penetrances for both a dominant and recessive mode of inheritance. Penetrances used for the dominant model were 0.01, 0.90 and 0.90 for the wild-type homozygote, mutant heterozygote and mutant homozygote, respectively. The recessive model was defined by penetrances 0.01, 0.01 and 0.80 for the wild-type homozygote, mutant heterozygote and mutant homozygote, respectively. A more complex model was also analysed using penetrances 0.01, 0.05, 0.30 for wild-type homozygotes, mutant heterozygotes and mutant homozygotes, respectively. For all models, the disease allele prevalence frequency assumed was of 0.001, and phenocopy rate of 1%. A parametric analysis assuming heterogeneity was computed using data from both families heterogeneity LOD (HLOD). A codominant allele frequency algorithm was used for the analysis, as suggested by the EasyLinkage user manual, for extended families. Following the initial scans, loci observed to have a high NPL and LOD score were further analysed using variable penetrances and phenocopy rates. These analyses were carried out for all the phenotypes described above, using the sex-averaged deCode genetic map.

Analysis of candidate gene

The eight exons of the tumour necrosis factor receptor-associated factor 6 (*TRAF6*) (MIM 602355) gene, including exon-intron boundaries and 1 kb upstream from the transcriptional start site, were amplified by PCR in two severely affected individuals and two normal individuals. Forward and reverse primers were designed using the reference sequence of the transcript ENST00000313105 (<http://www.ensembl.org>). Sequencing reactions were performed using the Big Dye Terminator Kit v3.1 and ABI 3130 genetic analyzer (PE Applied Biosystems, Foster City, CA), at MLS BioDNA Ltd, Malta.

Sequence variants identified were analysed in all family members and a cohort of unrelated postmenopausal women,^{17,18} either by direct sequencing or by restriction fragment length polymorphism (RFLP). PCR products (10 μ l) were digested using appropriate restriction enzymes according to the manufacturer's instructions (New England Biolabs, Beverly, MA, USA). Agarose gel electrophoresis was

carried out using 15 μ l of digested product. Following electrophoresis and staining with ethidium bromide, digested products were viewed and photographed under ultraviolet illumination (Polaroid DS-34). Nomenclature used refers to the actual nucleotide changes in the respective polymorphisms.

Results

The clinical and general characteristics of the individuals from both families are shown in Tables 1 and 2. A low BMD for chronological age phenotype was defined according to WHO criteria for an age- and sex-adjusted z-score of less than -1.0 at the LS and/or FN. Pedigree 1 is shown in Figure 1, with individual II:1 as the proband, whose recruited siblings were all found to be affected, with the most severe individual (II:8) having lumbar *t*-score and z-score of -5.68 and -3.09 , respectively. All daughters of the proband had very low BMD. Her 35-year-old daughter (III:2) was found to have lumbar *t*-score and z-score of -2.49 and -2.29 , respectively. Pedigree 2 (Figure 2) was identified from a 55-year-old woman (II:1), who was osteoporotic at the LS (*t*-score -3.47). Her youngest sister (II:6) was found to be severely osteoporotic at both the lumbar and FN with *t*-scores of -3.75 and -2.72 , respectively. Her other two sisters (II:4 and II:5) were also found to be severely osteoporotic but had z-scores > -1.0 . Low BMD was observed to occur even in males within this family. The 29-year-old son of the proband (III:1) had a lumbar *t*-score of -2.00 and z-score of -2.02 . His 37-year-old cousin (III:10) was also severely affected for his age having a lumbar *t*-score of -2.32 and z-score of -2.19 .

From the initial genome-wide scan in both families, evidence of linkage was observed to marker D11S1392 where a NPL score of 5.77 ($P=0.0006$) was obtained. A maximum HLOD score of 2.55 for this region was reached

Table 2 General characteristics of affected vs normal family members

Variable	Affected (n = 18)	Unaffected (n = 9)
Males/females (n)	6/12	6/3
Age (years)	48.6 \pm 15.8	46.4 \pm 16.9
BMI (kg/m ²)	25.7 \pm 3.5	25.9 \pm 4.8
Weight (kg)	62.9 \pm 11.4	68.0 \pm 13.0
Height (cm)	156.0 \pm 8.7	162.5 \pm 11.1
Ionised calcium (mmol/l)	1.28 \pm 0.07	1.29 \pm 0.02
LS BMD (g/cm ²)	0.795 \pm 0.137	1.018 \pm 0.187
LS <i>t</i> -score	-2.71 ± 1.18	-0.73 ± 1.66
LS z-score	-1.76 ± 0.49	0.17 ± 1.10
FN BMD (g/cm ²)	0.746 \pm 0.098	0.889 \pm 0.149
FN <i>t</i> -score	-1.68 ± 0.71	-0.53 ± 1.11
FN z-score	-0.97 ± 0.67	0.28 ± 0.78
Trochanter BMD (g/cm ²)	0.624 \pm 0.104	0.799 \pm 0.141
Ward's triangle BMD (g/cm ²)	0.567 \pm 0.092	0.707 \pm 0.175

Affected status was defined by LS and/or FN z-score of < -1.0 .

for the dominant mode of inheritance with 90% penetrance and phenocopy rate of 1%.

The same marker showed evidence of linkage when analysing families according to their femoral status (scenario V). For this phenotype, an NPL of 5.24 ($P=0.0020$) and an HLOD of 2.12 was obtained for this marker assuming a dominant mode of inheritance with 70% penetrance. This was the only locus that showed evidence of linkage to this phenotype with an affected status defined by femoral z-scores only.

Marker D5S1960 also showed evidence of linkage when a recessive mode of inheritance with 80% penetrance was analysed. Highest HLOD for this model was of 2.78 with a NPL of 5.10 ($P=0.0031$).

High-resolution scan

A high-resolution scan was performed by adding four additional markers at 1–2 cM intervals at the indicated locus on chromosome 11 (D11S4101, D11S935, D11S4102 and D11S1911). Linkage was confirmed to this region on chromosome 11, where an HLOD of 3.07, NPL of 7.0 ($P=0.0014$) and Zlr score of 3.01, were obtained to marker D11S935 that is 52.94 cM from 11p-telomere (in scenario 1). Figure 3 shows the LOD plot following fine mapping where the critical interval was narrowed after the addition of markers in this region. Table 3 shows the contribution of each individual family whereas the results for both families are shown in Table 4. Consistent results were obtained at this region on chromosome 11p12 with a highest Zlr score of 3.74 in scenarios IV and IX.

This locus was further analysed by varying the penetrance and phenocopy rates for the dominant mode of inheritance. Results obtained are shown in Table 5, where it can be observed that the highest HLOD score was of 3.32 at penetrances of 0.8 and 0.7 with a phenocopy rate of 5%.

When performing the same analysis on pedigrees assuming clinically unaffected individuals as having an unknown phenotype (scenarios VI–X), highest HLOD and NPL scores obtained were of 3.35 and 6.9 ($P=0.0002$), respectively, to marker D11S935 for the dominant mode of inheritance with 90% penetrance and phenocopy 1% (scenario IX).

TRAF6 sequencing

Candidate genes found within the linkage interval were selected with prior knowledge of bone physiology using the NCBI map viewer (<http://www.ncbi.nlm.nih.gov>) and freely available web tool The GeneSeeker v2.0 (<http://www.cmbi.kun.nl/GeneSeeker/>). The whole area from 49 to 55 cM on chromosome 11 (deCode genetic map) was searched for possible genes that might be involved in the disease. A total of 22 genes and two hypothetical proteins are known to be found in this region, with the best candidate being the gene coding for the TRAF6 (MIM

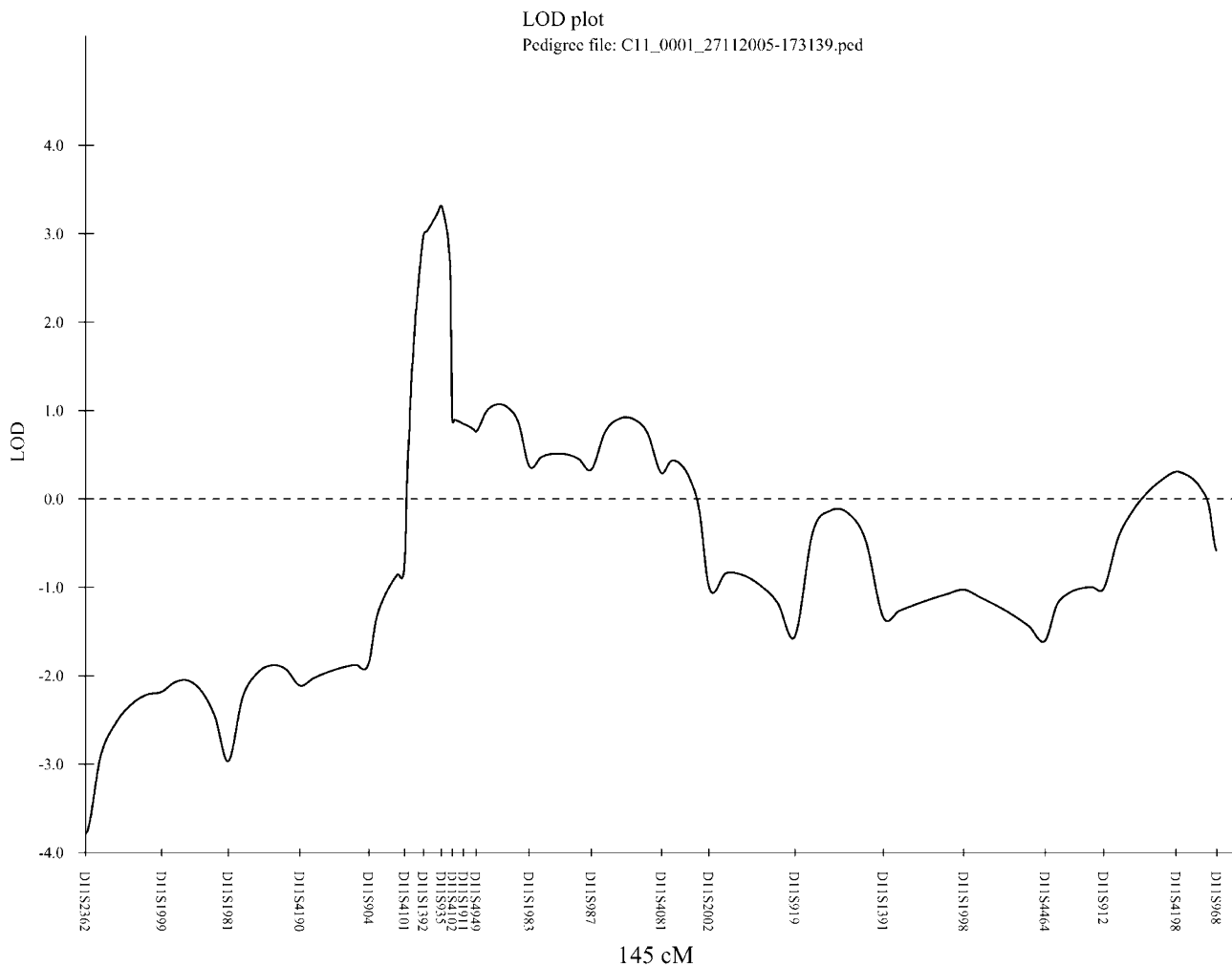


Figure 3 Analysis of Chromosome 11 dominant mode of inheritance (penetrance 0.80; phenocopy 0.05) following fine mapping.

602355), that is approximately 1 cM away from marker D11S935.

Following direct sequencing, three different variants were identified when compared to reference sequences on the NCBI and Ensembl databases. An A–T transversion was identified at position –721 (5' upstream of exon 1), when compared to TRAF6 reference sequence (AY228337) (Figure 4). This variant was not described previously. Following sequencing of all family members, three affected individuals from pedigree 1 were observed to be heterozygous for this variant. Individuals from pedigree 2 were all wild-type homozygotes. RFLP was carried out in 82 unrelated postmenopausal women, using restriction enzyme *HindIII*, which was able to cut the PCR product in the presence of the T allele. This variant was observed to be very rare within an unrelated group of postmenopausal women, as only three heterozygotes were observed. After screening 350 chromosomes in a random sample from the

general population, only two alleles were observed (0.57%) with this variant having a population frequency of 1.1%.

An already known insertion/deletion of a T in the intron between exons 4 and 5, in the polyT region 16 bp ahead of the exon–intron boundary (rs3830511), was identified. When analysing all family members and controls, only three individuals were observed to be heterozygotes for this insertion/deletion, one of whom was severely affected and the other two were normal individuals.

A transition from G to A was found in the intron between exons 6 and 7, 110 bp upstream of the exon–intron boundary. When sequencing all members from both families, four heterozygotes for this variant were identified, the rest were homozygous for the wild-type allele G. Three of the four heterozygous individuals for this variant were affected individuals, two of whom were also heterozygous for the T insertion/deletion described above. Genotyping by RFLP (*PvuII*) was performed in 82 unrelated

Table 3 Analysis of chromosome 11 for pedigrees 1 and 2 after fine mapping

Phenotype ^a	Pedigree 1			Pedigree 2		
	LOD (cM) ^b	NPL (P-values)	Zlr	LOD (cM) ^b	NPL (P-values)	Zlr
Scenario I	1.92 (54.35)	6.26 (0.0078)	2.84	1.21 (52.02)	4.41 (0.0098)	2.41
Scenario II	0.24 (55.77)	3.10 (0.0313)	2.12	0.00 (52.94)	3.06 (0.0625)	2.11
Scenario III	1.92 (54.35)	6.26 (0.0078)	2.84	1.11 (52.02)	4.74 (0.0117)	2.27
Scenario IV	1.64 (54.35)	4.41 (0.0156)	2.58	1.22 (52.94)	5.85 (0.0156)	2.75
Scenario V	0.40 (48.21)	1.94 (0.1250)	1.63	1.00 (50.64)	5.83 (0.0156)	2.88
Scenario VI	1.92 (54.35)	6.26 (0.0078)	2.84	1.04 (52.48)	4.42 (0.0098)	2.41
Scenario VII	1.35 (55.77)	3.10 (0.0313)	2.12	1.18 (52.94)	3.06 (0.0625)	2.11
Scenario VIII	1.92 (54.35)	6.26 (0.0078)	2.84	1.04 (51.56)	4.74 (0.0117)	2.27
Scenario IX	1.64 (54.35)	4.41 (0.0156)	2.58	1.77 (52.94)	5.85 (0.0156)	2.75
Scenario X	0.86 (48.21)	1.94 (0.1250)	1.63	1.75 (50.64)	5.83 (0.0156)	2.88

LOD scores shown are for the autosomal-dominant model with 90% penetrance and 1% phenocopy rate.

^aFor detailed description of phenotype refer to Materials and methods (phenotype definition). Phenotype definitions for scenarios VI–X were the same as scenarios I–V, but assuming unaffected individuals as having an unknown phenotype.

^bdeCode map position in parentheses in cM.

Table 4 Analysis of chromosome 11 for both pedigrees using an autosomal-dominant model after fine mapping

Phenotype	LOD (cM)	HLOD (α)	NPL (P-values)	Zlr
Scenario I	3.07 (52.94)	3.07 (1.00)	7.00 (0.0014)	3.01
Scenario II	-0.19 (52.94)	-0.00 (0.00)	4.02 (0.0038)	2.90
Scenario III	2.97 (52.94)	2.97 (1.00)	7.23 (0.0013)	3.04
Scenario IV	2.80 (52.94)	2.80 (1.00)	6.90 (0.0002)	3.74
Scenario V	1.26 (50.64)	1.26 (1.00)	5.24 (0.0020)	3.28
Scenario VI	2.90 (52.94)	2.90 (1.00)	7.00 (0.0014)	3.01
Scenario VII	2.46 (52.94)	2.46 (1.00)	4.02 (0.0038)	2.90
Scenario VIII	2.89 (52.94)	2.89 (1.00)	7.23 (0.0013)	3.04
Scenario IX	3.35 (52.94)	3.35 (1.00)	6.90 (0.0002)	3.74
Scenario X	2.59 (52.94)	2.59 (1.00)	5.37 (0.0020)	3.28

LOD scores shown are for the autosomal-dominant model with 90% penetrance and 1% phenocopy rate.

For detailed description of phenotype refer to Materials and methods (phenotype definition). Phenotype definitions for scenarios VI–X were the same as scenarios I–V, but assuming unaffected individuals as having an unknown phenotype.

deCode map position in parentheses in cM.

Table 5 Multipoint HLOD scores on chromosome 11 at 52.94 cM (D11S935) under an autosomal dominant model with variable penetrance and phenocopy

Penetrance	LOD/HLOD phenocopy = 1%	LOD/HLOD phenocopy = 5%	LOD/HLOD phenocopy = 10%	LOD/HLOD phenocopy = 15%	LOD/HLOD phenocopy = 20%
0.9	3.07	3.25	3.05	2.78	2.47
0.8	3.10	3.32	3.11	2.82	2.44
0.7	3.12	3.32	3.08	2.73	2.23
0.6	3.14	3.30	2.99	2.54	1.80
0.5	3.17	3.25	2.84	2.17	1.07

Only results obtained for scenario I are shown in this table.

postmenopausal women. Genotype frequencies observed were 72.3% GG, 26.5% GA and 1.2% AA.

Discussion

In this study, a novel locus on chromosome 11 was linked with osteoporosis in two extended Maltese families. Following fine mapping, the critical region was reduced to a 4 cM region at 11p12 between markers D11S1392 and

D11S4102. As shown in Figures 1 and 2, inherited haplotypes at this locus can be observed in both individual families. This seems to be inherited in an autosomal dominant fashion in affected individuals, with a number of recombination events occurring very close to this region. In pedigree 2 (Figure 2), an affected male (III:4) does not carry this causative haplotype suggesting that low BMD in this individual might be due to an environmental

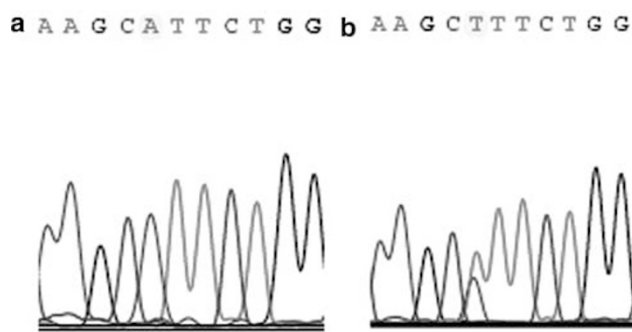


Figure 4 Electropherograms showing variant in TRAF6 promoter in a (a) normal control and (b) osteoporosis patient (sequence variant is shown highlighted).

factor (phenocopy). On the other hand, the suspected haplotype is present in two apparently clinically normal individuals (III:2 and III:7). This could be due to the fact that individual III:2 is only 23 years old and perhaps is still too young to be affected. Individual III:7 has a high BMI that might be modifying the effect of the causative gene. Also, other genetic and/or environmental factors might be modifying the effect of the causative gene in this region leading to incomplete penetrance of the disease allele. By assuming a reduced penetrance and increased phenocopy rate, more significant results were obtained at this locus (Table 5).

From this study, it is evident that multiple genes are involved in the pathogenesis of osteoporosis in these two families. Besides the most significant region on chromosome 11p12, another region on chromosome 5 gave an NPL of 5.10 and HLOD of 2.78 to marker D5S1960. Other interesting regions that require further investigation were found on chromosomes 3, 6, 9 and 17. Linkage to chromosomes 3q and 17q22 was also reported by Koller *et al*,¹³ where strong evidence of linkage to chromosome 17q22 was reported with variations in femoral structure. Among the candidate genes involved in bone physiology on chromosome 17q22 are those coding for type I collagen (*COL1A1*) and noggin.²³

No evidence of linkage was observed to previously indicated regions such as those on chromosomes 11q12–13, 1p36,^{5–10} 20p12,¹⁵ 11p23 and Xq27.¹⁶ Weak evidence of linkage with whole body BMD was observed by Wilson *et al*⁴ to a region that is 10 cM away from our indicated region on chromosome 11p12, which is approximately 20 cM away from 11q12–13 indicated in previous studies which is well known to contain genes responsible for other bone phenotypes,^{9,10} such as those coding for the low-density lipoprotein receptor-related protein 5 and the T-cell immune regulator 1.

In a multifactorial disease, such as osteoporosis, lack of concordance between different studies might be due to a multitude of environmental factors including dietary and

lifestyle factors, possibly modifying the effect of the causative gene. Apart from this, linkage analysis is affected by incomplete penetrance, phenocopy and genetic heterogeneity, where different genes might be responsible for disease in different ethnic groups.

Differences in study design, approach and analysis of data that might result in type I errors, are other reasons for lack of replication of results. Our study is the second published study using a qualitative instead of a quantitative type of analysis for osteoporosis. This approach has also been used by Styrkarsdottir *et al*¹⁵ in a large study performed in the Icelandic population, where variants within the *BMP-2* gene on chromosome 20p12 were associated with the disease. In previous studies, linkage was most often sought by using BMD measurements at different anatomical sites as a QTL.^{3–7,11–14,16} This approach is not always advantageous especially as it is known that BMD is not the only determinant of osteoporosis and fracture risk. Each individual trait, such as BMD and other radiographic measurements is more likely to be affected by a number of loci, each having a very small effect on the phenotype. This makes it very difficult to detect linkage unless a larger sample size is used. However, when different genome scans for a number of complex diseases were compared for evaluation, it was shown that highly significant linkage was obtained from studies using a qualitative approach and significant results were not always achieved by the largest studies.²⁴

Our study was carried out using only two large pedigrees in which osteoporosis appeared to exhibit classical Mendelian inheritance. Other loci were linked to other human diseases in studies using single extended families with multiple affected individuals such as those for an autosomal dominant keratoconus²⁵ and for the localisation of a cholesterol-lowering gene.²⁶ An appropriate study design is very important for the successful localisation of genes responsible for a genetically heterogeneous disease such as osteoporosis. For heterogeneous disease, power is increased by using large extended families especially those coming from an isolated population rather than from an outbred one.²⁷ Two examples of isolated populations are the Icelandic and Finnish, where in the latter a number of genes and chromosomal loci were confirmed.²⁸ The *BMP2* gene on chromosome 20p12 was identified in a study carried out in the isolated population of Iceland.¹⁵

The observation that common haplotypes were found in all affected members of individual families together with the high HLOD of 3.32 and NPL score of 7.0, points to a novel locus for osteoporosis on 11p12. This locus has been also linked to a number of inflammatory disorders. Amos *et al*²⁹ showed evidence of linkage to 11p12 with rheumatoid arthritis in 642 Caucasian families. Apart from rheumatoid arthritis, this locus was also linked with coeliac disease³⁰ and inflammatory bowel disease.³¹ Such observations might support the hypothesis that a common

inflammatory process controlled by genes found at this locus might be responsible for the onset of these diseases. This indicates that osteoporosis might also be caused by a mild subclinical inflammation that is triggered with advancing age.³²

TRAF6 plays a very important role in the differentiation and activation of osteoclasts when it interacts with receptor activator of NF- κ B (RANK) and activates transcription mediated by activator protein-1.^{33,34} The importance of TRAF6 in osteoclast function was shown from experiments on knockout mice where osteopetrosis was observed in TRAF6-deficient mice.³⁵ The RANKL/RANK system and therefore TRAF6 plays a very important role in what is now known as osteoimmunology. Takayanagi³⁶ describes how immunology and bone physiology interact and share various molecules. TRAF6 together with other TRAFs are recruited by RANK upon stimulation by RANKL, which is also expressed by T cells, and controlled by signal transducers and activators of transcription through signalling from the interferon- γ receptor. From our DNA sequencing analysis, we identified three sequence variants in the *TRAF6* gene, one in the promoter region and the other two in intervening introns. The variant found in the promoter region was detected in three affected individuals in one family and was observed to be very rare in the population. Further investigation of this variant is being conducted to address its possible role on gene expression.

No variants were found in the coding regions of TRAF6, showing that its sequence is conserved, thus indicating its important biological role. Recently, Lappalainen *et al*³⁷ identified an insertion/deletion in a polyT stretch in intron 3, a similar polymorphism to the one we identified in intron 4, but failed to find association with inflammatory bowel disease. As these polymorphisms are found in the polyT region 3' upstream from the intron-exon boundary, one cannot exclude their possible role in the splicing mechanism. Sequence variants identified in this study do not seem to be completely linked with the disease in these two families. This does not completely exclude its possible role in the pathogenesis of osteoporosis, as other variants might be present in regions that were not sequenced (introns). Also different genes might be responsible for the disease in each individual family. This might be suggested by highest LOD scores for pedigree 1 being closer to the *TRAF6* gene (~54 cM), whereas those observed in pedigree 2 were approximately 2 cM upstream (~52 cM) (Table 3). Also different alleles were observed in the two inherited haplotypes in the two families. Another possible candidate gene found closer to the 52 cM region is the gene coding for CD44, which was also observed to play a role in inflammatory bone loss.³⁸

These results suggest a possible role of a gene on chromosome 11p12 that might be responsible for osteoporosis in these families. Sequence variants within the *TRAF6* gene and other genes found at this locus might be

responsible for this type of osteoporosis. Further studies of this region will help us to understand better the pathophysiology of osteoporosis and may lead to the development of more effective treatments.

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Web Resources and Software

Online Mendelian Inheritance in Man (OMIM) <http://www.ncbi.nlm.nih.gov/Omim/> (search for osteoporosis, TRAF6)
Ensembl Gene Database (<http://www.ensembl.org>)
GeneSeeker v2.0 (<http://www.cmbi.kun.nl/GeneSeeker/>) searching for genes expressed in bones, osteoblasts, osteoclasts and skeleton
The software Cyrillic v2.1.3 (Cherwell Scientific Publishing Ltd) was used to design pedigrees in Figures 1 and 2.

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