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ORIGINAL ARTICLE

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Assessment of linkage and association of 13 genetic loci with bone mineral density

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Abstract Bone mineral density (BMD), an important risk factor for osteoporosis, is a complex trait likely affected by multiple genes. The linkage and/or association of 13 polymorphic loci of seven candidate genes (estrogen receptor alpha [*ERα*] and beta [*ERβ*], calcium-sensing receptor, vitamin D receptor, collagen type 1α1, low-density lipoprotein [LDL] receptor-related protein 5 [*LRPS*], and transforming growth factor β1) were evaluated in 177 southern Chinese pedigrees of 674 subjects, with each pedigree identified through a proband having a BMD Z score of –1.28 or less at the hip or spine. A suggestive linkage was detected between the IVS1-351A/G polymorphism of *ERα* and spine BMD, and between the 1082G/A, 1730G/A, and D14S1026 polymorphisms of *ERβ* and BMD at both spine and hip. The quantitative transmission disequilibrium test (QTDT) detected total family association between 1730G/A of *ERβ* and BMD at spine and hip; between D14S1026 of *ERβ* and hip BMD; and between the 266A/G and 2220C/T polymorphisms of *LRP5* and hip BMD. Similar total family associations were detected when only the females were analyzed. In addition, the IVS1-397T/C polymorphism of *ERα* was associated with spine BMD, and the 266A/G and 2220C/T polymorphisms of *LRP5* were associated with femoral neck BMD in the females. A within-family association was detected with the IVS1-397T/C polymorphism of *ERα*, and the 266A/G and 2220C/T polymorphisms of *LRP5* in the females. The effect of each polymorphism on BMD variance ranged from 1% to 4%. In conclusion, *ERα*, *ERβ* and *LRP5* are important candidate genes determining BMD variation, especially in females.

Key words BMD · candidate genes · association · linkage

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Introduction

Osteoporosis is a common disease characterized by compromised bone strength and increased susceptibility to fracture [1]. Extensive evidence by Slemenda et al. [2] and Gueguen et al. [3] has shown that bone mineral density (BMD), an important risk factor for osteoporosis, is under strong genetic control with heritability estimates that range between 0.5 and 0.9. As reviewed by Albagha and Ralston [4], a number of studies showed the BMD is likely to be controlled by multiple genes and influenced by environmental and lifestyle factors, perhaps each contributing a small effect to the final BMD determination. Numerous studies, by Koller et al. [5,6], Duncan et al. [7], Deng et al. [8], Karasik et al. [9], Kammerer et al. [10], Wilson et al. [11], Econs et al. [12], Ralston et al. [13], and Devoto et al. [14], trying to identify genes for BMD determination showed variable results. Similarly, case-control association studies were confounded by a number of problems, making interpretation of the results difficult. It is well known that, with the association study approach, false-positive results may arise due to population admixture. On the other hand, the linkage approach for a complex trait is often limited by small sample size and inadequate statistical power [15]. An alternative approach, the transmission disequilibrium test

(TDT), developed by Spielman et al. [16] for the analysis of complex diseases, is not affected by the problem of population admixture or stratification and has been applied for studying continuously distributed quantitative traits such as BMD [17,18].

A number of genes have been postulated to affect BMD determination, but the role and effect size of these genes is unclear. In this study, we attempted to evaluate the linkage and association of 13 polymorphic loci in seven candidate genes as putative quantitative trait loci (QTLs) for BMD variation in families with a proband having low BMD. The candidate genes studied were estrogen receptor alpha (*ERα*) and beta (*ERβ*), calcium-sensing receptor (*CASR*), vitamin D receptor (*VDR*), collagen type 1α1 (*COL1A1*), low-density lipoprotein [LDL] receptor-related protein 5 (*LRP5*), and the transforming growth factor β1 (*TGFβ1*) gene.

Subjects and methods

Subjects

Community-dwelling subjects of southern Chinese descent were enrolled in the study to determine the genetic and environmental risk factors for osteoporosis. These subjects were recruited when they passed by road shows and health talks on osteoporosis held in various districts of Hong Kong between 1998 and 2002. They were invited to the Osteoporosis Centre at Queen Mary Hospital, the University of Hong Kong, for BMD measurement by dual-energy X-ray absorptiometry (DXA; Hologic QDR 4500 plus; Hologic, Waltham, MA, USA). To increase the power of detecting QTL, subjects with BMD in the lowest tenth centile of the population, equivalent to a Z score of –1.28 or less at either the lumbar spine (L1-4) or hip region were identified as the probands of this family study, and their extended family members were also invited to participate. Individuals with disease known to affect bone metabolism, premature menopause (age < 40 years), bilateral oophorectomy, or drug use that could affect bone turnover and BMD were excluded. All subjects underwent a physical examination and were interviewed by a trained research assistant, using a structured questionnaire for their ethnicity; social, medical, and reproductive histories; dietary and lifestyle factors; and family history of osteoporosis. Dietary calcium and phytoestrogen intakes were determined by a semi-quantitative food frequency questionnaire, as described by Mei et al. [19]. Physical activity including sports-related and non-sports leisure activity was recorded. A total activity score was obtained from the sum of scores in these two domains to give a total score ranging from 0 (low physical activity) to 4 (high physical activity). All participants gave their informed consent and the study was approved by the Ethics Committee of the University of Hong Kong and conducted according to the Declaration of Helsinki.

Measurement of bone size, mass, and density

BMD (g/cm^2) values at the L1-4 lumbar spine, femoral neck, trochanter, and total hip were measured by DXA (Hologic QDR 4500 plus; Hologic). The in-vivo precision of the machine for the lumbar spine, femoral neck, and total hip region was 1.2%, 1.5%, and 1.5%, respectively [20]. BMD Z scores (SD from the mean of sex-and-age matched controls) were determined from local reference data [21,22].

Genotyping

Genomic DNA was extracted from peripheral blood leukocytes by a standard procedure. The genes studied were *CASR*, *COL1A1*, *ERα* and *ERβ*, *LRP5*, *TGFβ1*, and *VDR* (Table 1). Genotypes of 13 polymorphic sites of the seven candidate genes were determined by a polymerase chain reaction (PCR)-based method for each participant.

CASR

Genotyping of the (CA)n repeat polymorphism in the flanking region of the *CASR* gene was performed as described by Tsukamoto et al. [23]. The oligonucleotide primers used for PCR are shown in Table 1. The PCR products were separated by electrophoresis on an ABI PRISM 3700 genetic analyzer (Applied Biosystems, Foster City, CA, USA). Gel was analyzed using GeneScan and Genotyper softwares (Applied Biosystems).

COL1A1

To identify the polymorphic sites of the promoter region of the *COLIA1* gene in our population, DNA samples from 50 normal southern Chinese were studied. We amplified 3000 base pairs (bp) of the upstream region of *COL1A1* by PCR and directly sequenced them using the ABI PRISM 3700 genetic analyzer. Two polymorphic sites, at –1363C/G and –1997G/T, of the *COL1A1* gene were studied. Genotyping of –1363C/G was done by using the ABI PRISM 7700 Sequence Detection System with TaqMan Universal PCR Master Mix and Assays-on-Demand Gene expression probes (Applied Biosystems). The reaction mixture of 10 µl included 5 µl 2× TaqMan PCR Master Mix (Applied Biosystems), 0.25 µl Assays–by-Design Gene expression probes (Applied Biosystems), 20 ng of genomic DNA in 0.25 µl Tris-EDTA (TE) buffer, and 4.5 µl double-distilled H_2O. Cycling conditions for PCR consisted of a preincubation period of 2 min at 50°C, and an initial hot start for 10 min at 95°C, followed by 45 cycles of denaturation at 95°C for 15 s and annealing at extension at 64°C for 60 s.

The polymorphic site -1997G/T was genotyped by a PCR-restriction fragment length polymorphism (PCR-RFLP)-based method, as described by Garcia-Giralt et al. [24]. DNA samples were amplified with the primers described in Table 1 and subsequently digested with restric-

Table 1. Studied genes and primers for genotyping

Candidate gene	Chromosome	Variation	Primer sequences	Reference
CASR	3q21-q24	CA repeat	5′-TAATGTTGCTATGAACATTTTATG-3′ 5′-GAACTTGGGGTCCTCCATAG-3′	Tsukamoto et al.[23] 2000
COLIA1	17q21.3-q22.1	-1363C/G -1997G/T	 5′-CACCCTGCCCTAGACCAC-3′ 5′-GAAAATATAGAGTTTCCAGAG-3′	Garcia-Giralt et al.[24] 2002
ERα	6q25.1	IVS1-397T/C IVS1-351A/G	5′-TATCCAGGGTTATGTGGCAA-3′ 5′-AAAATGACAAAATGAAATTAGCTGG-3′	Kobayashi et al.[25] 1996
ERβ	14q23	1082G/A	5′-TCTTGCTTTCCCCAGGCTTT-3′ 5′-ACCTGTCCAGAACAAGATCT-3′	Sundarrajan et al.[26] 2001
		1730G/A	5′-TTTTTGTCCCCATAGTAACA-3′ 5′-AATGAGGGACCACAGCA-3′	
		D14S1026, CA repeat	5′-GGTAAACCATGGTCTGTACC-3′ 5′-AACAAAATGTTGAATGAGTGGG-3′	Lau et al.[27] 2002
LRP5	11q13.4	266A/G	5′-TCTGGGCATAGTGCTCCATC-3′ 5′-TTCCGGGATGTGCCATTGAG-3′	Okubo et al.[29] 2002
		2220C/T	5′-GGATGGGCAAGAACCTCTACTG-3′ 5′-ACGAGGACTTGCCGGAACT-3′	
		3989T/C	5′-GACTGTCAGGACCGCTCACACG-3′ 5′-AAGGTTTTCAGAGCCCCTAC-3′	
*TGFβ*1	19q13.2	29C/T	5′-CCCACCACACCAGCCCTGTTC 5′-TTCCGCTTCACCAGCTCCATGT	Ziv et al.[30] 2003
VDR	12q13.11	2C/T	5′-AGCTGGCCCTGGCACTGACTCTGCTCT-3′ 5′-ACCTGTCCAGAACAAGATCT-3′	Gross et al.[31] 1996

tion enzyme *Eco*31I. Digestion products were electrophoresed in 2% agarose gels.

ERα and *ERβ*

IVS1-397T/C (*Pvu*II polymorphism) and IVS1-351A/G (*Xba*I polymorphism) of the *ERα* gene were genotyped by a PCR-RFLP method as described by Kobayashi et al. [25].

For the 1082G/A and 1730G/A polymorphisms of the *ERβ* gene, genotyping was performed by PCR-RFLP methods, as described by Sundarrajan et al. [26]. For D14S1026, the (CA)n repeat polymorphism in the flanking region of the *ERβ* gene, a fragment of intron 5 in the *ERβ* gene was amplified using 5′ labeled fluorescent forward primer, as described by Lau et al. [27] and Ogawa et al. [28]. The products were analyzed using the automatic ABI PRISM 3700 genetic analyser, as described above.

LRP5

To identify polymorphic sites in the *LRP5* gene in southern Chinese, the 23 exons and their adjacent intronic region of the *LRP5* gene were sequenced in 60 Chinese women (kindly performed by Professor P.Y. Kwok, UCLA, University of California, USA). The association of three single-nucleotide polymorphisms (SNPs), 266A/G, 2220C/T and 3989T/C, with BMD was analyzed.

For the 266A/G and 3989T/C polymorphisms, genotyping was performed by PCR-RFLP methods, as described by Okubo et al. [29]. For the 2220C/T polymorphism, genotyping was determined by allelic discrimination, using TaqMan Universal PCR Master Mix and Assays-by-Design Gene expression probes (Applied Biosystems). The following Assays–by-Design Gene expression probes were used: VIC-ACCAACAGAATCGAA and FAM-TGGGACCAATAGAAT. The total reaction mixture of 10 µl included 5 µl 2× TaqMan PCR Master Mix, 0.25 µl Assays-by-Design Gene expression probes, 20 ng of genomic DNA in 0.25 µl TE buffer, and 4.5 µl double-distilled H_2O. The PCR conditions used were as for the *COLIA1* gene described above. The ABI Prism 7700 Sequence Detector was used for data acquisition.

TGFβI

For the 29C/T polymorphism of the *TGFβI* gene, genotype was determined by allelic discrimination, using TaqMan Universal PCR Master Mix and Assays-by-Design Gene expression probes (Applied Biosystems) as described by Ziv et al. [30]. The following Assays-by-Design Gene expression probes were used: FAM-TAGCAGCAGCAGCAGCAGCCGC for the T allele and VIC-TAGCAGCAGCGGCAGCAGCCG for the C allele.

VDR

For the 2C/T polymorphisms of the *VDR* gene, genotyping was performed by PCR-RFLP methods, as shown by Gross et al. [31]. The PCR product, a 265-bp fragment of exon 1 in the *VDR* gene, was digested with *FokI* restriction enzyme and electrophoresed in 2% agarose gel.

Table 2. Clinical characteristics of the study population of 674 individuals in 177 families

	Total	Men	Women
Subject number	674	107	567
Age (years)	49.0 ± 15.7	52.8 ± 17.0	48.3 ± 15.4
Height (cm)	156.6 ± 0.1	167.6 ± 0.1	154.5 ± 0.1
Weight (kg)	54.1 ± 9.4	65.8 ± 9.4	52.0 ± 7.7
Menarche age (years)	–	–	13.6 ± 1.9
Age at menopause (years)	–	–	49.1 ± 3.9[a]
Calcium intake (mg/day)	627.1 ± 243.9	668.3 ± 271.9	619.4 ± 237.8
Isoflavone intake (mg/day)	30.4 ± 32.7	34.6 ± 44.4	29.6 ± 30.0
Smoker (%)	30 (4.3%)	15 (14%)	15 (2.6%)
Drinker (%)	41 (5.9%)	17 (15.9%)	24 (4.2%)
Exercise score	3.46 ± 0.65	3.48 ± 0.67	3.48 ± 0.67
Spine BMD (g/cm^2)	0.840 ± 0.145	0.873 ± 0.145	0.833 ± 0.144
Femoral neck BMD (g/cm^2)	0.644 ± 0.110	0.685 ± 0.101	0.636 ± 0.104
Trochanter BMD (g/cm^2)	0.556 ± 0.101	0.608 ± 0.101	0.547 ± 0.098
Total hip BMD (g/cm^2)	0.736 ± 0.120	0.811 ± 0.124	0.722 ± 0.114

Data values are expressed as means ± SD
[a] Two hundred and eighty-eight women were postmenopausal

Statistical analysis

Multipoint variance components linkage analysis was done using Merlin-Regress, a regression-based method for selected samples, as described by Abecasis et al. [32]; it is available at http://www.sph.umich.edu/csg/abecasis/Merlin. BMD Z-scores at the lumbar spine, trochanter, femoral neck, and total hip were used as the phenotype.

Association analysis in the pedigree was performed by quantitative transmission disequilibrium test (QTDT). Using the QTDT program, tests of population stratification, within-family association, and total association (including within- and between-family components) between each of the genetic markers and BMD at the spine and hip region were performed. The tests implemented in the QTDT program (available on the internet http://www.sph.umich.edu/csg/abecasis/QTDT/) were developed under a variance component framework [18]. Association effects are divided into between- and within-family components. The within-family component is robust to population stratification and is significant only in the presence of linkage disequilibrium (LD) due to close linkage. The between-family component is sensitive to population stratification; thus, the total association test can only be applied if population stratification is absent. The test of population stratification is to evaluate whether the between-family component is equal to the within-family component.

To increase the genetic signal-to-noise ratio and to decrease the proportion of the residual phenotypic variation attributable to random environmental factors, BMD values were adjusted for possible covariates that were identified in the stepwise regression model to affect BMD independently. Also, to better clarify the effect of sex on BMD, analysis was also performed on the females alone, with the males being eliminated from the analysis by indicating their phenotypes as unknown. Because there were only 107 males in the sample and the results may not have been reliable, the males were not analyzed separately.

The QTDT program generates *P* values for various tests via asymptotic χ^2 distribution. The empirical significance level is reported for the within-family association test and was computed from 1000 Monte Carlo permutations, as described by McIntyre et al. [33]. In the present study, a result with $P < 0.005$ for the individual analysis was regarded as significant, and a *P* value ranging from 0.005 to 0.05 was considered as suggestive or weak evidence. Another way to correct for multiple testing is Bonferroni correction. However, as shown by McIntyre et al. [33], it is thought to be too conservative for associated alleles at linked markers, resulting in a loss of power. Computation of empirical *P* values should reduce associations attributable to type I error. The effect size of the major allele in comparison to the minor allele of any significant gene is reported. Similarly, the effect size of the significant CA repeat allele in comparison to other CA repeats is reported. The effect sizes are reported as beta-coefficients in the Table 5.

Results

One hundred and seventy-seven pedigrees with 674 subjects (107 males, 567 females) from two to three generations were analyzed. The pedigrees ranged in size from 3 to 15 individuals, with a mean of 5.2 individuals. There were 482 parent-offspring pairs, 467 sib-pairs, 14 grandparent-grandchild pairs, and 43 cousin pairs. The clinical characteristics of the subjects are summarized in Table 2. The mean age of the subjects was 49.0 ± 15.7 (mean ± SD) years. Among the females, 288 (51%) were postmenopausal and the mean age at menopause was 49.1 ± 3.9 years. The mean age at menarche of all female subjects was 13.6 ± 1.9 years. The average dietary intakes of calcium and isoflavone were 625 mg and 30 mg, respectively.

A stepwise regression model revealed that, among the anthropometric and environmental factors, the important independent variables affecting BMD were sex, age, height, and weight. Other lifestyle factors, such as dietary calcium and phytoestrogen intake, physical activity, and smoking and drinking habits were not predictive of BMD.

In all subsequent analysis, BMD values were adjusted for sex, age, height, and weight.

The genotype and allele frequencies, as well as the *P* values of the Hardy-Weinberg equilibrium test, are summarized in Table 3. The results of linkage analysis are reported in Table 4. After adjusting for sex, age, height and weight, evidence of linkage was detected at the polymorphic sites of both the *ERα* and *ERβ* genes. For the *ERα* gene, the IVS1-351A/G polymorphism was in suggestive linkage with spine BMD (Log-of-odds [LOD] = 0.67; *P* = 0.04). As for the *ERβ* gene, all three polymorphic loci were in linkage with BMD. Suggestive linkages were found between 1082G/A and femoral neck BMD (LOD = 1.25; *P* = 0.008), total hip BMD (LOD = 0.78; *P* = 0.03), and spine BMD (LOD = 0.72; *P* = 0.03); between 1730G/A and femoral neck BMD (LOD = 0.98; *P* = 0.02), and spine BMD (LOD = 0.64; *P* = 0.04); and between D14S1026 and femoral neck BMD (LOD = 1.78; *P* = 0.002), total hip BMD (LOD = 0.79; *P* = 0.03), and spine BMD (LOD = 0.62; *P* = 0.05).

Table 5 shows the results of the QTDT analyses of all families. For total association, the 1730G/A polymorphism of the *ERβ* gene was associated with BMD variation at the spine and hip, with *P* values less than 0.05. The G allele of this polymorphism had a negative influence on BMD, with an effect size around –2% to –3%. The 156-bp allele (*P* = 0.02) and the 168-bp allele (*P* = 0.04) of D14S1026, the CA dinucleotide repeat polymorphism of the *ERβ* gene, were found to be associated with femoral neck BMD. An opposite influence was found between these two CA repeat polymorphisms, with the 156-bp allele having a negative effect (effect size, –2%) while the 168-bp allele had a positive effect on BMD (effect size, 3.6%). The 266A/G polymorphism of the *LRP5* gene was found to be associated with hip BMD, including femoral neck (*P* = 0.03), trochanter (*P* = 0.04), and total hip (*P* = 0.04). The A allele of this polymorphism had a positive influence on BMD, with an effect size of 2%.

Table 6 shows the results of the QTDT analyses on the females alone. For the total association, the 1730G/A polymorphism of the *ERβ* gene was associated with BMD (*P* = 0.02) at the femoral neck. The G allele was associated with a negative influence on BMD, with an effect size of –2%. The 156-bp allele of D14S1026 was found to be associated with BMD at all three sites of the hip region (*P* < 0.05). This polymorphism had a negative influence on BMD, with an effect size around –1% to –2%. The 166-bp and 168-bp alleles of D14S1026 were found to be associated with femoral neck BMD (both *P* < 0.05), with a positive effect of 2% and 4%, respectively. The *ERα* IVSI-397T/C polymorphism showed significant within-family association with spine BMD (*P* = 0.02). For the 266A/G polymorphism of *LRP5*, it was found to be associated with femoral neck BMD (total association, *P* = 0.03). The A allele of this polymorphism had a positive influence, with an effect size of 2%. The 2220C/T polymorphism of *LRP5* showed total (*P* = 0.03) and within-family association (*P* = 0.004) with trochanter and femoral neck BMD. The C allele of this polymorphism had a positive influence on BMD, with an effect size around 2%. Also, the T allele of the 3989T/C polymorphism of *LRP5* was shown to be positively associated with femoral neck BMD (within-family association, *P* = 0.03), with an effect size of 2%.

Discussion

In the present study, 13 polymorphic sites were assessed for putative QTLs for BMD variation in southern Chinese families having a proband with low BMD. Using different analytical approaches, both the *ERα* and *ERβ* genes were shown to have greatest evidence of linkage and/or association with BMD. These polymorphic sites included the IVS1-397T/C (*Pvu*II) and IVS1-351A/G (*Xba*I polymorphisms) of the *ERα* gene; and the D14S1026, 1082G/A (*Rsa*I), and 1730G/A (*Alu*I) polymorphisms of the *ERβ* gene. In addition, the 266A/G, 2220C/T and 3989T/C polymorphisms of *LRP5* were shown to be associated with BMD when all families were analyzed and when only the females were studied.

In view of the importance of estrogen signalling in bone and the high prevalence of osteoporosis in postmenopausal women, numerous studies, by Kobayashi et al. [25], Lau et al. [27], Ogawa et al. [28], Sano et al. [34], Albagha et al. [35], Qin et al. [36], Khosla et al. [37], Ioannidis et al. [38,39], Arko et al. [40], and Shearman et al. [41], have examined the association of *ERα* and *ERβ* gene polymorphisms and BMD, as well as fracture risk. In the present study, linkage was documented between the *ERα* IVS1-351A/G polymorphism and spine BMD. The association between *ERα* gene polymorphism and BMD was first reported by Sano et al. [34], who demonstrated the association between a TA repeat polymorphism in the *ERα* gene promoter and BMD in a small number of Japanese women. Other investigators, including Kobayashi et al. [25], Albagha et al. [35], Qin et al. [36], and Khosla et al. [37] have reported the association between the *Pvu*II (IVS1-397T/C) and *Xba*I (IVS1-351A/G) polymorphisms of the *ERα* gene and BMD, as well as the rate of bone loss. Our finding of linkage of the IVS1-351A/G polymorphism with spine BMD is consistent with the metaanalysis by Ioannidis et al. [38] on 5834 Caucasian women, which revealed that homozygote subjects for the XX genotype had higher BMD when compared to the Xx and xx genotypes, whereas the *Pvu*II polymorphism was not associated with BMD. However, another recent metaanalysis, also by Ioannidis et al. [39], of 18917 individuals from eight European centers, revealed that *Xba*I determined fracture risk by mechanisms that are independent of BMD. Zhao et al. [42] recently reported the association of two other SNPs, rs932477 and rs2228480, of the *ERα* gene with femoral neck BMD in Caucasian nuclear families, and the SNP haplotype TCGCGGG was associated with higher BMD at the spine. However, after adjusting for multiple testing, these associations did not reach statistical significance.

The IVS1-351A/G and IVS1-397T/C polymorphisms are intronic, and, so far, the functional significance of these polymorphic alleles is unclear. However, introns may

Table 3. Allele and genotype frequencies and *P* values for Hardy-Weinberg equilibrium (HWE) test

	Genotype frequency (%)	Allele frequency HWE (%)	*P* value
COL1A1 (-1997G/T)			
GG	54.7	G = 74.1	0.70
GT	38.9	C = 25.9	
TT	6.1		
COL1A1 (-1363C/G)			
CC	47.3	C = 68.9	0.91
GC	43.1	G = 31.1	
GG	9.6		
ERα (IVS1-397T/C)			
TT	34.1	T = 58.8	0.65
CT	49.3	C = 41.2	
CC	16.6		
ERα (IVS1-351A/G)			
AA	55.8	A = 75.6	0.21
GA	39.5	G = 24.5	
GG	4.7		
ERβ (1082G/A)			
GG	37.7	G = 60.5	0.23
GA	45.6	A = 39.5	
AA	16.7		
ERβ (1730G/A)			
GG	86.1	G = 92.8	0.83
AG	13.4	A = 7.2	
AA	0.5		
LRP5 (266A/G)			
AA	84.8	A = 92.1	0.95
AG	14.6	G = 7.9	
GG	0.6		
LRP5 (2220C/T)			
CC	67.1	C = 81.7	0.45
CT	29.1	T = 18.3	
TT	3.8		
LRP5 (3989T/C)			
TT	68.0	T = 81.5	0.21
CT	28.0	C = 18.5	
CC	4.0		
TGFβ1 (29C/T)			
CC	39.6	C = 62.5	0.53
CT	45.8	T = 37.5	
TT	14.6		
VDR (2C/T)			
CC	25.7	C = 51.4	0.52
CT	51.3	T = 48.6	
TT	23.0		
CASR (allele size, bp)	CA repeat number		
220	12.0	57.4	
222	13.0	0.3	
226	15.0	1.0	
228	16.0	0.2	
230	17.0	1.8	
232	18.0	39.0	
236	20.0	0.1	
238	21.0	0.1	
ERβ (D14S1026, allele size, bp)	CA repeat number		
146	16.0	0.1	
148	17.0	0.1	
150	18.0	0.7	
152	19.0	11.3	
154	20.0	28.2	
156	21.0	10.5	
158	22.0	2.6	
160	23.0	11.3	
162	24.0	9.3	
164	25.0	16.2	
166	26.0	7.3	
168	27.0	2.0	
170	28.0	0.2	

Table 4. Linkage analyses of the studied genotypes and BMD Z scores at spine and hip region by Merlin-Regress

Polymorphic site	Spine		Femoral neck		Trochanter		Total hip	
	LOD	*P*	LOD	*P*	LOD	*P*	LOD	*P*
COLIA1 promoter-1997G/T	0.02	0.4	0.00	0.5	0.00	0.5	0.04	0.3
COLIA1 promoter-1363C/G	0.01	0.4	0.00	0.5	0.00	0.5	0.01	0.4
ERα IVS1-397T/C	0.16	0.2	0.00	0.5	0.01	0.4	0.05	0.3
ERα IVS1-351A/G	0.67	**0.04**	0.01	0.4	0.00	0.5	0.02	0.4
ERβ 1082G/A	0.72	**0.03**	1.25	**0.008**	0.31	0.12	0.78	**0.03**
ERβ 1730G/A	0.64	**0.04**	0.98	**0.02**	0.27	0.13	0.52	0.06
ERβ, D14S1026	0.62	**0.05**	1.78	**0.002**	0.45	0.07	0.79	**0.03**
LRP5 266A/G	0.00	0.5	0.01	0.4	0.00	0.5	0.02	0.4
LRP5 2220C/T	0.03	0.4	0.02	0.4	0.00	0.5	0.03	0.4
LRP5 3989T/C	0.00	0.5	0.02	0.4	0.00	0.5	0.01	0.4
TGFβ1 29C/T	0.03	0.3	0.01	0.4	0.05	0.3	0.44	0.08
VDR 2C/T	0.02	0.4	0.15	0.2	0.13	0.2	0.06	0.3
CASR (CA) repeat	0.03	0.4	0.21	0.2	0.00	0.5	0.01	0.4

Table 5. Association analyses of the studied genotypes and BMD at the spine and hip region, using the QTDT program

Polymorphic site	Spine			Femoral neck			Trochanter			Total hip		
	χ^2	β	SD	χ^2	β	SD	χ^2	β	SD	χ^2	β	SD
Total association												
ERβ 1730G/A	3.87*	–0.027	0.014	7.38**	–0.026	0.010	4.51*	–0.019	0.009	5.54*	–0.025	0.011
ERβ, D14S1026												
156 bp $(CA)_{21}$	1.46	0	0	5.76*	–0.020	0.008	1.81	0	0	3.00	0	0
168 bp $(CA)_{27}$	0.44	0	0	4.14*	0.036	0.018	2.70	0	0	2.81	0	0
LRP5 266A/G	2.98	0	0	4.71*	0.020	0.009	4.19*	0.018	0.009	4.17*	0.021	0.01

* $P < 0.05$; ** $P < 0.01$

Data values were adjusted for sex, age, height, and weight. Only the results of tests with $P \leq 0.05$ are listed here

Table 6. Association analyses of the studied genotypes and BMD at the spine and hip region for females, using the QTDT program

Polymorphic site	Spine			Femoral neck			Trochanter			Total hip		
	χ^2	β	SD	χ^2	β	SD	χ^2	β	SD	χ^2	β	SD
Total association												
ERβ 1730G/A	3.59	0	0	5.37*	–0.024	0.010	3.05	0	0	3.21	0	0
ERβ, D14S1026												
156 bp $(CA)_{21}$	0.04	0	0	6.81**	–0.023	0.001	3.85*	–0.016	0.001	5.28*	–0.022	0.001
166 bp $(CA)_{26}$	0.12	0	0	4.24*	0.022	0.011	3.51	0	0	1.99	0	0
168 bp $(CA)_{27}$	0	0	0	4.69*	0.042	0.019	1.93	0	0	2.31	0	0
LRP5 266A/G	1.05	0	0	4.81*	0.023	0.010	3.05	0	0	3.24	0	0
LRP5 2220C/T	2.41	0	0	1.89	0	0	4.50*	0.014	0.001	1.48	0	0
Within-family association[a]												
ERα IVS1-397T/C	4.08*	0.027	0.013	0.09	0	0	1.96	0	0	3.82	0	0
LRP5 2220C/T	1.98	0	0	4.13**	0.024	0.012	1.31	0	0	0.43	0	0
LRP5 3989T/C	2.24	0	0	4.02*	0.022	0.011	0.73	0	0	0.26	0	0

* $P < 0.05$; ** $P < 0.01$

Data values were adjusted for age, height, and weight. Only the results of tests with $P \leq 0.05$ are listed here

[a] *P* values are the empirical *P* values from 1000 permutation tests

contain regulatory elements that affect gene transcription or translation. For example, as shown by Herrington et al. [43], the IVS1-397T/C polymorphic site is located within a B-myb binding site and may potentially have regulatory effects on *ERα* gene function. Alternatively, these polymorphic sites may be in linkage disequilibrium with other variants that affect receptor structure or, more likely, messenger RNA and protein expression.

By simultaneously providing evidence for association and linkage using various tests, our study confirms that the *ERβ* gene is a QTL for BMD. D14S1026, and the CA dinucleotide repeats, 1082G/A and 1730G/A polymorphisms

of *ERβ*, were in linkage and/or association with spine and hip BMD. The recently published genome-wide linkage analysis from the Framingham study by Karasik et al. [9] showed evidence of a suggestive linkage of spine BMD at chromosome 14q21.3, where the *ERβ* locus is located. The first study revealing significant association of the CA dinucleotide repeat polymorphisms of *ERβ* with spine BMD was reported by Ogawa et al. [28] in a cohort of postmenopausal Japanese women. We previously reported association of the CA dinucleotide repeat polymorphism of *ERβ* with both spine and femoral neck BMD in pre- but not postmenopausal women [27]. A G-to-A substitution in exon 5 of *ERβ* was examined by Arko et al. [40] in a small sample of postmenopausal Slovenian women, but no significant association was noted. In the Framingham study, Shearman et al. [41] reported that two SNPs in the intronic region as well as the CA repeat polymorphism of *ERβ* were found to be significantly associated with hip BMD. However, another SNP, the 1730G/A polymorphism reported by Khosla et al. [37], was not related to bone loss in a longitudinal study of Caucasian subjects.

D14S1026, the CA dinucleotide repeat polymorphism, was located in intron 5 of the *ERβ* gene and was in strong linkage disequilibrium with the two SNPs that we studied, i.e. nucleotide (nt) 1082G/A in exon 5 and 1730G/A in exon 8. However, as shown by Sundarrajan et al. [26], these two polymorphic alleles are not associated with any alteration of the amino-acid sequence of the *ERβ* protein. In the present study, shorter CA repeats were associated with a negative effect, while longer repeats were associated with a positive effect on BMD. It is tempting to postulate that the repeat size may affect the folding of the *ERβ* protein and alter the binding and, hence, the bioavailabity of estrogen.

Similarly to other studies, we noted differential effects of these polymorphic markers on BMD at specific skeletal sites. Indeed, site-specific differences in the heritability of BMD and bone size are being increasingly appreciated. Whole-genome studies by Koller et al. [6], Karasik et al. [9], Ralston et al. [13], Devoto et al. [14], Enmark et al. [44], and Deng et al. [45] also revealed linkage of different chromosomal regions to BMD at the spine and hip. These observations highlighted the complexity of genetic interplay with local and environmental factors to determine the final BMD variance at specific sites.

In the present study, we failed to show significant linkage and/or association between BMD variation and some candidate loci of *CASR*, *COL1A1*, and *TGFβ1*, which were reported to be of importance in Caucasian populations. Apart from ethnic differences, absence of linkage between these loci and BMD variation could possibly be explained by the weak contribution of these loci to BMD variation or by the limited power of the study to detect genes with a small effect. To enhance the statistical power of our study, we had ascertained families with a proband having BMD at the lowest tenth centile of the population. Based on the "TDT for threshold-selected quantitative traits" program of the Genetic Power Calculator (http://pngu.mgh.harvard.edu/~purcell/gpc/qtdt.html), this study actually has more than 80% power to detect a QTL that explains 5% of the BMD variation if the minor allele frequency is greater than 3%. Another possibility for the negative findings could be due to the choice of SNPs evaluated in this study. The SNPs studied were either previously reported to be associated with BMD in other populations, or they were located in the 5′upstream region of the gene, e.g., *COL1A1*, or in the exonic region associated with changes in amino-acid sequence, e.g., *LRP5*. Using such an approach, we could not evaluate all SNP haplotype blocks of each candidate gene and, hence, we could have missed relevant SNPs that were related to BMD determination.

In conclusion, among the seven candidate genes studied, evidence of linkage and association with BMD was shown with the *ERα* and *ERβ* genes. In addition, the *LRP5* gene was also found to be associated with BMD. By identifying the genes responsible for the pathogenesis of osteoporosis, new therapeutic products can be devised, and treatment can be better tailored or targeted depending on the genetic background of the subjects. Genetic studies may also allow a better identification of at-risk subjects who could be targeted for early preventive measures before significant bone loss occurs.

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