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Association of candidate gene polymorphisms with bone mineral density in community-dwelling Japanese women and men

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Abstract. Although bone mineral density (BMD) is a complex trait that is influenced by both genetic and environmental factors, heritability studies in twins and families have shown that genetic factors account for 60-85% of the variance in BMD. We examined the relations of six candidate gene polymorphisms to BMD in community-dwelling women and men. The 2238 subjects (1110 women, 1128 men) were aged 40-79 years and were randomly recruited to a population-based prospective cohort study of aging and age-related diseases in Japan. BMD at the distal and proximal radius was measured by peripheral quantitative computed tomography, and BMD for the total body, lumbar spine (L2-L4), right femoral neck, and right trochanter was measured by dual-energy X-ray absorptiometry. Genotypes for the 1019C→T (Pro319Ser) polymorphism of *GJA4* and the 1462A→G (Lys469Glu) polymorphism of *ICAM1* were determined with a fluorescence-based allele-specific DNA primer assay system, and those for the 386G→A (Ala99Thr) polymorphism of *PLOD1*, the A→G polymorphism of *CNR2*, the 1583G→A (Arg528Lys) polymorphism of *ALAP*, and the -514C→T polymorphism of *LIPC* were determined by melting curve analysis. The polymorphisms of *ALAP* and *PLOD1* were associated with BMD in premenopausal women; those of *ICAM1* and *LIPC* with BMD in postmenopausal women; that of *CNR2* with BMD in premenopausal and postmenopausal women; and that of *GJA4* with BMD in men. Among these polymorphisms, those of *ICAM1*, *CNR2*, and *GJA4* were markedly associated with BMD. These results suggest that *ALAP*, *PLOD1*, *ICAM1*, *LIPC*, and *CNR2* are susceptibility loci for reduced bone mass in Japanese women and that *GJA4* constitutes such a locus in Japanese men. The polymorphisms of *ICAM1* and *CNR2* may confer susceptibility to postmenopausal osteoporosis in women, and that of *GJA4* to osteoporosis in men.

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Introduction

Osteoporosis, a major health problem of the elderly, is characterized by a reduction in bone mineral density (BMD) and a deterioration in the microarchitecture of bone, both of which result in predisposition to fractures (1). Although reproductive, nutritional, and lifestyle factors influence BMD, family and twin studies have suggested that BMD is largely (60-85%) heritable and under the control of multiple genes (2-4). Personalized prevention of osteoporosis and osteoporotic fractures is an important public health goal, for which one approach is to identify disease susceptibility genes. Although genetic linkage analyses (5-7) and candidate gene association studies (7-10) have implicated various loci and genes in predisposition to osteoporosis or fractures, the genes that confer susceptibility to this disease remain to be identified definitively. In addition, because of ethnic differences in gene polymorphisms as well as in lifestyle and other environmental factors, it is important to examine polymorphisms related to BMD in each ethnic group.

We have been attempting to identify genes significantly associated with BMD in Japanese women or men with a population-based approach. In the present study, we selected six candidate genes that might be expected to contribute to bone remodeling (Table I) and examined the relations of polymorphisms of these genes to BMD, even though there is no apparent biological link among these genes. Our aim was to identify a single polymorphism significantly associated with BMD for each gene. Among several polymorphisms previously identified, we selected those that might be expected to affect gene function. We thus examined the relations of these polymorphisms to BMD in community-dwelling Japanese women and men.

Materials and methods

Study population. The National Institute for Longevity Sciences - Longitudinal Study of Aging is a population-based prospective cohort study of aging and age-related diseases, the details of which have been described previously (11-15). Individuals with disorders known to cause abnormalities of bone metabolism, including diabetes mellitus, chronic renal failure, rheumatoid arthritis, as well as thyroid, parathyroid, adrenal, and other endocrine diseases, or those who had taken drugs that affect bone metabolism, such as estrogen,

Table I. The six gene polymorphisms examined in the present study.

Locus	Gene	Symbol	Polymorphism	dbSNP
1p36.3-36.2	Procollagen-lysine, 2-oxoglutarate 5-dioxygenase	*PLOD1*	386G→A (Ala99Thr)	rs7551175
1p36.11	Cannabinoid receptor 2	*CNR2*	A→G	rs2501431
1p35.1	Gap junction protein, α-4	*GJA4*	1019C→T (Pro319Ser)	rs1764391
5q15	Adipocyte-derived leucine aminopeptidase	*ALAP*	1583G→A (Arg528Lys)	rs30187
15q21-23	Lipase, hepatic	*LIPC*	-514C→T	rs1800588
19p13.3-13.2	Intercellular adhesion molecule 1	*ICAM1*	1462A→G (Lys469Glu)	rs5498

glucocorticoids, bisphosphonates, and vitamin D, were excluded from the present study. We thus examined the relations of gene polymorphisms to BMD in 2238 individuals (1110 women and 1128 men). Individuals whose genotypes were not successfully determined were also excluded from the analysis. In addition, to uncover potential differences between women according to menopausal status, we conducted all analyses for premenopausal and postmenopausal women separately. Menopausal status was evaluated with a detailed questionnaire, and menopause was defined as complete cessation of menstruation. Because of their small number (n=19), perimenopausal women were excluded from this analysis. The study protocol complied with the Declaration of Helsinki and was approved by the Committee on Ethics of Human Research of the National Institute for Longevity Sciences. Written informed consent was obtained from each subject.

Measurement of BMD. BMD at the radius was measured by peripheral quantitative computed tomography (pQCT) with a Desiscan 1000 instrument (Scanco Medical, Bassersdorf, Switzerland) and was expressed as D50 (BMD for the inner 50% of the cross-sectional area of the distal radius, comprising mostly cancellous bone), D100 (BMD for the entire cross-sectional area of the distal radius, including both cancellous and cortical bone), and P100 (BMD for the entire cross-sectional area of the proximal radius, consisting mostly of cortical bone). BMD for the total body, lumbar spine (L2-L4), right femoral neck, and right trochanter was measured by dual-energy X-ray absorptiometry (DXA) with a QDR 4500 instrument (Hologic, Bedford, MA, USA). The coefficients of variation of the pQCT instrument for BMD values were 0.7% (D50), 1.0% (D100), and 0.6% (P100), and those of the DXA instrument were 0.9% (total body), 0.9% (L2-L4), 1.3% (femoral neck), and 1.0% (trochanter).

Determination of genotype. Genotypes for polymorphisms of *GJA4* and *ICAM1* were determined with a fluorescence-based allele-specific DNA primer assay system (Toyobo Gene Analysis, Tsuruga, Japan) (16). Primers and other conditions for genotyping are shown in Table II. The polymorphic region of each gene was amplified by the polymerase chain reaction (PCR) with allele-specific sense primers labeled at the 5' end with either fluorescein isothiocyanate (FITC) or Texas red and with an antisense primer labeled at the 5' end with biotin. The reaction mixture (25 μl) contained 20 ng of DNA, 5 pmol of each primer, 0.2 mmol/l of each deoxynucleoside triphosphate, 2.5 or 4.5 mmol/l $MgCl_2$, and 1 U of rTaq DNA polymerase (Toyobo, Osaka, Japan) in polymerase buffer. The amplification protocol comprised an initial denaturation at 95˚C for 5 min; 35 cycles of denaturation at 95˚C for 30 sec, annealing at 60˚C for 30 sec, and extension at 72˚C for 30 sec; and a final extension at 72˚C for 2 min. The amplified DNA was incubated with streptavidin-conjugated magnetic beads in the wells of a 96-well plate at room temperature, and the plate was then placed on a magnetic stand. The supernatants from each well were transferred to the wells of a 96-well plate containing 0.01 mol/l NaOH and were measured for fluorescence with a microplate reader (Fluoroscan Ascent; Dainippon Pharmaceutical, Osaka, Japan) at excitation and emission wavelengths of 485 and 538 nm, respectively, for FITC; and of 584 and 612 nm, respectively, for Texas red.

Genotypes for polymorphisms of *PLOD1*, *CNR2*, *ALAP*, and *LIPC* were determined by melting curve analysis (intercalater-mediated fluorescence resonance energy transfer probe method). The polymorphic region of each gene was amplified by PCR (Table II) in a reaction mixture (25 μl) containing 20 ng of DNA, 5 pmol of each primer, 0.2 mmol/l of each deoxynucleoside triphosphate, 2 or 3 mmol/l $MgCl_2$, and 1.25 U of rTaq DNA polymerase in polymerase buffer. The amplification protocol comprised an initial denaturation at 95˚C for 5 min; 45 or 50 cycles of denaturation at 95˚C for 30 sec, annealing at 65˚C for 30 sec, and extension at 72˚C for 30 sec; and a final extension at 72˚C for 2 min. A mixture (2 μl) of 10 pmol of probe and SYBR-Green was added to the PCR products, which were then transferred to a PRISM 7700 instrument (Applied Biosystems, Foster City, CA, USA) for measurement of melting temperature. The program for analytic melting comprised incubation at 95˚C for 30 sec, 40˚C for 1 min, and temperatures increasing to 80˚C over 10 min. The fluorescence signals were detected at excitation and emission wavelengths of 485 and 612 nm, respectively.

Statistical analysis. Data are presented as means ± SE. Statistical analysis was performed with SAS software (SAS Institute, Cary, NC, USA). Data were compared among three genotype groups by one-way analysis of variance and the Tukey-Kramer *post hoc* test, and between two groups (dominant or recessive model) by the unpaired Student's t-test. BMD values were compared among genotypes for each polymorphism with adjustment for age, height, and body weight by the least squares method in a general linear model. Allele frequencies were estimated by the gene-counting

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Primers, probes, and other PCR conditions for genotyping.

Gene	Polymorphism	Sense primer with FITC	Sense primer with Texas red
GJA4	1019C→T (Pro319Ser)	CCTCAGAATGGCCAAAAxTC	CTCAGAATGGCCAAAAxCC
ICAM1	1462A→G (Lys469Glu)	AAGGGGAGGTCACCCGxGA	AGGGGAGGTCACCCGxAA

	Antisense primer with biotin	Annealing (˚C)	Cycles	Mg^{2+} (mM)
GJA4	GCAGAGCTGCTGGGACGA	60	35	2.5
ICAM1	CTCACAGAGCACATTCACGGTCAC	60	35	4.5

Gene	Polymorphism	Sense primer	Antisense primer
PLOD1	386G→A (Ala99Thr)	AAGCACGCAGACAAGGAGGATCTG	GAGGGCCTCATTTTAGAATATTCTTCTATCTTC
CNR2	A→G	GGGCAGGTAGGAGACTAGTGCTGAGAG	CTCACCCGTGGAAGGGCACTG
ALAP	1583G→A (Arg528Lys)	CCCTTCATGTAGTGCTCTTGCTTCATG	GCATCAGGAAGGGGTGGATGTG
LIPC	-514C→T	GGGCATCTTTGCTTCTTCGTCAG	TTGGTGATGCTTGTGGTCAAAGTG

	Probe	Annealing (˚C)	Cycles	Mg^{2+} (mM)
PLOD1	CATTCTCTTGGCAGACAGGTAG	65	45	2.0
CNR2	CACATGATGCCCAGGGTC	65	45	2.0
ALAP	CCCCTCTGCAGTGTCCAA	65	45	2.0
LIPC	TTCACCCCCATGTCAAAA	65	50	3.0

All primer and probe sequences are 5'→3'.

method, and the Chi-square test was used to identify significant departure from Hardy-Weinberg equilibrium. A P value of <0.05 was considered statistically significant.

Results

Relation of the 386G→A (Ala99Thr) polymorphism of PLOD1 to BMD. The distribution of 386G→A genotypes of *PLOD1* was in Hardy-Weinberg equilibrium, and age, height, and body weight did not differ among genotypes, for all women (Table III) or for premenopausal or postmenopausal women. Among all women, BMD for the femoral neck, with adjustment for age, height, and body weight, was greater in individuals with the *GG* genotype than in those with the *GA* genotype or in the combined group of *GA* and *AA* genotypes (Table III). BMD for the trochanter was also greater in individuals with the *GG* genotype than in the combined group of *GA* and *AA* genotypes. The differences in BMD for

Table III. BMD and other characteristics for all women (n=1109) according to the *PLOD1* genotype.[a]

Characteristic	*GG*	*GA*	*AA*	*GG + GA*	*GA + AA*
Number (%)	621 (56.0)	427 (38.5)	61 (5.5)	1048 (94.5)	488 (44.0)
Age (years)	59.3±0.4	59.0±0.5	60.8±1.4	59.2±0.3	59.3±0.5
Height (cm)	151.2±0.2	151.4±0.3	151.3±0.8	151.3±0.2	151.4±0.3
Body weight (kg)	52.7±0.3	52.4±0.4	53.2±1.0	52.6±0.3	52.5±0.4
BMD measured with pQCT (mg/cm^3)					
D50	186.3±2.5	183.5±3.0	184.4±7.9	185.2±1.9	183.6±2.8
D100	485.2±3.6	486.8±4.3	484.8±11.4	485.9±2.8	486.5±4.1
P100	1158.0±5.8	1146.3±7.0	1164.1±18.3	1153.3±4.5	1148.6±6.5
BMD measured with DXA (g/cm^2)					
Total body	0.968±0.003	0.960±0.004	0.965±0.011	0.966±0.003	0.961±0.004
L2-L4	0.868±0.005	0.861±0.006	0.863±0.016	0.865±0.004	0.861±0.006
Femoral neck	0.683±0.003	0.670±0.004[b]	0.684±0.011	0.677±0.003	0.671±0.004[c]
Trochanter	0.576±0.003	0.564±0.004	0.569±0.010	0.571±0.003	0.565±0.004[d]

[a]BMD is adjusted for age, height, and body weight. Data are means ± SE. [b]P=0.0442, [c]P=0.0320, [d]P=0.0266 versus *GG*.

Table IV. BMD and other characteristics for all women (n=1106) according to the *CNR2* genotype.[a]

Characteristic	*AA*	*AG*	*GG*	*AA + AG*	*AG + GG*
Number (%)	402 (36.3)	544 (49.2)	160 (14.5)	946 (85.5)	704 (63.7)
Age (years)	59.1±0.5	59.1±0.5	60.4±0.9	59.1±0.4	59.4±0.4
Height (cm)	151.6±0.3	151.4±0.3	150.5±0.5	151.5±0.2	151.2±0.2
Body weight (kg)	52.8±0.4[b]	53.0±0.4[c]	51.0±0.6	52.9±0.3[d]	52.5±0.3
BMD measured with pQCT (mg/cm^3)					
D50	192.9±3.1[e]	182.1±2.6[f]	176.3±4.9	186.7±2.0	180.8±2.3[g]
D100	491.4±4.5	484.8±3.8	476.7±7.1	487.6±2.9	483.0±3.4
P100	1154.0±7.2	1157.3±6.2	1140.5±11.5	1155.9±4.7	1153.5±5.4
BMD measured with DXA (g/cm^2)					
Total body	0.971±0.004[h]	0.965±0.004	0.952±0.007	0.967±0.003[i]	0.962±0.003
L2-L4	0.876±0.006[j]	0.863±0.005	0.846±0.010	0.869±0.004[k]	0.859±0.005[l]
Femoral neck	0.682±0.004	0.676±0.004	0.673±0.007	0.679±0.003	0.675±0.003
Trochanter	0.575±0.004	0.571±0.004	0.559±0.006	0.573±0.003	0.569±0.003

[a]BMD is adjusted for age, height, and body weight. Data are means ± SE. [b]P=0.0497, [c]P=0.0218, [d]P=0.0070, [e]P=0.0118, [h]P=0.0449, [i]P=0.0352, [j]P=0.0352, [k]P=0.0412 versus *GG*; [f]P=0.0220, [g]P=0.0018, [l]P=0.0382 versus *AA*.

the femoral neck and trochanter between individuals with the *GG* genotype and the combined group of *GA* and *AA* genotypes (expressed as a percentage of the larger value) were 1.8 and 1.9%, respectively. For premenopausal women, BMD for the femoral neck and that for the trochanter were greater in individuals with the *GG* genotype than in the combined group of *GA* and *AA* genotypes (data not shown). For postmenopausal women or for men, there was no difference in BMD among *PLOD1* genotypes (data not shown).

Relation of the A→G polymorphism of CNR2 to BMD. The distribution of A→G genotypes of *CNR2* was in Hardy-Weinberg equilibrium, and age and height did not differ among genotypes, for all women (Table IV). Body weight was greater in women with the *AA* genotype or the *AG* genotype or in the combined group of *AA* and *AG* genotypes than in women with the *GG* genotype. Among all women, BMD for D50 was greater in individuals with the *AA* genotype than in those with the *AG* genotype or the *GG* genotype or in the combined group of *AG* and *GG* genotypes (Table IV). BMD for the total body and that for the lumbar spine were greater in individuals with the *AA* genotype or in the combined group of *AA* and *AG* genotypes than in women with the *GG* genotype. BMD for the lumbar spine was also greater in individuals with the *AA* genotype than in the combined group of *AG* and *GG* genotypes. The differences in BMD for D50, total body, and lumbar spine between individuals with the *AA* genotype and those with the *GG* genotype were 8.6, 2.0, and 3.4%, respectively.

The distribution of A→G genotypes of *CNR2* was in Hardy-Weinberg equilibrium in premenopausal (Table V) and postmenopausal (Table VI) women. Age, height, or body weight did not differ among genotypes for premenopausal or postmenopausal women. For premenopausal women, BMD for D50 was greater in individuals with the *AA* genotype than in those with the *AG* genotype or in the combined group of *AG* and *GG* genotypes (Table V). BMD for the lumbar spine was also greater in individuals with the *AA* genotype than in the combined group of *AG* and *GG* genotypes. The differences in BMD for D50 and the lumbar spine between individuals with the *AA* genotype and the combined group of *AG* and *GG* genotypes were 6.4 and 2.7%, respectively.

For postmenopausal women, BMD for D50 was greater in individuals with the *AA* genotype or in the combined group of *AA* and *AG* genotypes than in individuals with the *GG* genotype, and was greater in individuals with the *AA* genotype than in the combined group of *AG* and *GG* genotypes (Table VI). BMD for the total body and that for the trochanter were greater in the combined group of *AA* and *AG* genotypes than in individuals with the *GG* genotype. The difference in BMD for D50 between individuals with the *AA* genotype and those with the *GG* genotype was 10.1%, and those for the total body and trochanter between the combined group of *AA* and *AG* genotypes and individuals with the *GG* genotype were 2.1 and 2.8%, respectively.

For men, the distribution of A→G genotypes of *CNR2* was in Hardy-Weinberg equilibrium, and BMD for the total body, femoral neck, or trochanter was greater in the combined group of *AG* and *GG* genotypes than in individuals with the *AA* genotype (data not shown).

Relation of the 1019C→T (Pro319Ser) polymorphism of GJA4 to BMD. The distribution of 1019C→T genotypes of *GJA4* was in Hardy-Weinberg equilibrium, and BMD for the femoral neck was greater in individuals with the *CT* genotype or in the combined group of *CT* and *TT* genotypes than in individuals with the *CC* genotype, for all women and for postmenopausal women (data not shown). For premenopausal women, there was no difference in BMD among *GJA4* genotypes (data not shown).

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BMD and other characteristics for premenopausal women (n=276) according to the *CNR2* genotype.[a]

Characteristic	*AA*	*AG*	*GG*	*AA + AG*	*AG + GG*
Number (%)	106 (38.4)	140 (50.7)	30 (10.9)	246 (89.1)	170 (61.6)
Age (years)	46.8±0.4	46.1±0.4	44.8±0.8	46.4±0.3	45.9±0.4
Height (cm)	154.3±0.5	154.6±0.4	154.3±0.9	154.5±0.3	154.5±0.4
Body weight (kg)	54.7±0.8	54.6±0.7	52.0±1.5	54.6±0.5	54.1±0.6
BMD measured with pQCT (mg/cm^3)					
D50	255.7±5.4	237.8±4.6[b]	246.4±10.1	245.3±3.6	239.3±4.2[c]
D100	614.5±7.7	598.7±6.6	612.3±14.3	605.4±5.0	601.1±6.0
P100	1366.3±11.4	1354.0±9.7	1370.9±21.2	1359.2±7.4	1356.9±8.8
BMD measured with DXA (g/cm^2)					
Total body	1.102±0.008	1.085±0.007	1.100±0.015	1.092±0.005	1.088±0.006
L2-L4	1.042±0.011	1.011±0.010	1.027±0.021	1.024±0.007	1.014±0.009[d]
Femoral neck	0.776±0.009	0.763±0.008	0.790±0.016	0.769±0.006	0.768±0.007
Trochanter	0.666±0.008	0.652±0.007	0.659±0.015	0.658±0.005	0.653±0.006

[a]BMD is adjusted for age, height, and body weight. Data are means ± SE. [b]P=0.0334, [c]P=0.0173, [d]P=0.0480 versus *AA*.

Table VI. BMD and other characteristics for postmenopausal women (n=813) according to the *CNR2* genotype.[a]

Characteristic	*AA*	*AG*	*GG*	*AA + AG*	*AG + GG*
Number (%)	291 (35.8)	393 (48.3)	129 (15.9)	684 (84.1)	522 (64.2)
Age (years)	63.7±0.5	63.9±0.4	64.1±0.8	63.8±0.3	64.0±0.4
Height (cm)	150.5±0.4	150.1±0.3	149.6±0.5	150.3±0.2	150.0±0.3
Body weight (kg)	52.1±0.5	52.3±0.4	50.9±0.7	52.2±0.3	52.0±0.4
BMD measured with pQCT (mg/cm^3)					
D50	170.9±3.7[b]	161.9±3.2	153.6±5.6	165.7±2.5[c]	159.8±2.8[d]
D100	447.3±5.4	443.9±4.6	434.0±8.1	445.4±3.5	441.5±4.0
P100	1078.2±8.9	1086.4±7.6	1066.4±13.5	1082.9±5.8	1081.5±6.6
BMD measured with DXA (g/cm^2)					
Total body	0.924±0.005	0.921±0.004	0.903±0.008	0.922±0.003[e]	0.917±0.004
L2-L4	0.817±0.007	0.810±0.006	0.789±0.011	0.813±0.005	0.805±0.006
Femoral neck	0.649±0.005	0.644±0.004	0.635±0.007	0.646±0.003	0.642±0.004
Trochanter	0.542±0.005	0.543±0.004	0.527±0.007	0.542±0.003[f]	0.539±0.004

[a]BMD is adjusted for age, height, and body weight. Data are means ± SE. [b]P=0.0290, [c]P=0.0497, [e]P=0.0237, [f]P=0.0445 versus *GG*; [d]P=0.0179 versus *AA*.

The distribution of 1019C→T genotypes of *GJA4* was in Hardy-Weinberg equilibrium, and age, height, or body weight did not differ among genotypes for men (Table VII). BMD for the total body was greater in individuals with the *CT* genotype or in the combined group of *CC* and *CT* genotypes than in individuals with the *TT* genotype. BMD for the lumbar spine and that for the femoral neck were greater in individuals with the *CC* genotype or the *CT* genotype or in the combined group of *CC* and *CT* genotypes than in individuals with the *TT* genotype. BMD for the trochanter was also greater in the combined group of *CC* and *CT* genotypes than in individuals with the *TT* genotype. The differences in BMD for the total body, lumbar spine, femoral neck, and trochanter between the combined group of *CC* and *CT* genotypes and individuals with the *TT* genotype were 3.1, 8.3, 6.4, and 4.6%, respectively.

Relation of the 1583G→A (Arg528Lys) polymorphism of ALAP to BMD. The distribution of 1583G→A genotypes of *ALAP* was in Hardy-Weinberg equilibrium, and age, height, and body weight did not differ among genotypes, for all women, premenopausal women (Table VIII), or postmenopausal women. For premenopausal women, BMD for the total body, femoral neck, or trochanter was greater in individuals with the *GG* genotype than in the combined group of *GA* and *AA* genotypes (Table VIII). The differences in BMD for the total body, femoral neck, and trochanter between individuals with the *GG* genotype and the combined

Table VII. BMD and other characteristics for all men (n=1128) according to the *GJA4* genotype.[a]

Characteristic	CC	CT	TT	CC + CT	CT + TT
Number (%)	729 (64.6)	357 (31.7)	42 (3.7)	1086 (96.3)	399 (35.4)
Age (years)	59.2±0.4	59.0±0.6	61.0±1.7	59.1±0.3	59.2±0.5
Height (cm)	164.5±0.2	164.8±0.3	164.8±1.0	164.6±0.2	164.8±0.3
Body weight (kg)	62.4±0.3	62.6±0.5	61.5±1.4	62.5±0.3	62.5±0.5
BMD measured with pQCT (mg/cm^3)					
D50	266.3±2.5	268.7±3.5	257.3±10.3	267.1±2.0	267.5±3.3
D100	541.2±3.4	541.9±4.9	533.4±14.2	541.4±2.8	541.0±4.6
P100	1184.3±5.2	1188.0±7.4	1185.8±21.6	1185.5±4.2	1187.8±7.0
BMD measured with DXA (g/cm^2)					
Total body	1.087±0.003	1.091±0.005[b]	1.055±0.014	1.089±0.003[c]	1.087±0.005
L2-L4	0.987±0.006[d]	0.983±0.008[e]	0.904±0.023	0.986±0.005[f]	0.975±0.007
Femoral neck	0.754±0.004[g]	0.758±0.005[h]	0.707±0.015	0.755±0.003[i]	0.753±0.005
Trochanter	0.669±0.004	0.671±0.005	0.639±0.015	0.670±0.003[j]	0.668±0.005

[a]BMD is adjusted for age, height, and body weight. Data are means ± SE. [b]P=0.0370, [c]P=0.0181, [d]P=0.0011, [e]P=0.0029, [f]P=0.0004, [g]P=0.0085, [h]P=0.0042, [i]P=0.0020, [j]P=0.0446 versus *TT*.

Table VIII. BMD and other characteristics for premenopausal women (n=276) according to the *ALAP* genotype.[a]

Characteristic	*GG*	*GA*	*AA*	*GG + GA*	*GA + AA*
Number (%)	80 (29.0)	127 (46.0)	69 (25.0)	207 (75.0)	196 (71.0)
Age (years)	46.4±0.5	45.9±0.4	46.7±0.6	46.1±0.3	46.2±0.3
Height (cm)	154.5±0.5	154.3±0.4	154.6±0.6	154.4±0.3	154.4±0.3
Body weight (kg)	54.2±0.9	54.8±0.7	53.5±1.0	54.6±0.6	54.4±0.6
BMD measured with pQCT (mg/cm^3)					
D50	250.7±6.2	241.8±5.0	247.6±6.6	245.3±3.9	243.9±4.0
D100	609.9±8.7	603.4±7.0	607.5±9.4	605.9±5.5	604.9±5.6
P100	1357.1±12.9	1359.2±10.4	1370.7±13.9	1358.4±8.1	1363.3±8.3
BMD measured with DXA (g/cm^2)					
Total body	1.113±0.009	1.086±0.007	1.085±0.010	1.097±0.006	1.086±0.006[b]
L2-L4	1.036±0.013	1.017±0.010	1.024±0.014	1.025±0.008	1.020±0.008
Femoral neck	0.789±0.010	0.761±0.008	0.771±0.011	0.772±0.006	0.764±0.006[c]
Trochanter	0.676±0.009	0.653±0.007	0.646±0.010	0.662±0.006	0.650±0.006[d]

[a]BMD is adjusted for age, height, and body weight. Data are means ± SE. [b]P=0.0107, [c]P=0.0380, [d]P=0.0191 versus *GG*.

group of *GA* and *AA* genotypes were 2.4, 3.2, and 3.8%, respectively. There was no difference in BMD among *ALAP* genotypes for all women or postmenopausal women (data not shown).

For men, the distribution of 1583G→A genotypes of *ALAP* was in Hardy-Weinberg equilibrium, and BMD for the femoral neck was greater in individuals with the *GG* genotype than in those with the *GA* genotype or in the combined group of *GA* and *AA* genotypes (data not shown).

Relation of the -514C→T polymorphism of LIPC to BMD. The distribution of -514C→T genotypes of *LIPC* was in Hardy-Weinberg equilibrium, and age, height, and body weight did not differ among genotypes, for all women (Table IX), premenopausal women, or postmenopausal women (Table X). For all women, BMD for D50 was greater in the combined group of *CC* and *CT* genotypes than in individuals with the *TT* genotype (Table IX). BMD for the total body was greater in individuals with the *CT* genotype than in those with the *CC* genotype or the *TT* genotype, and was greater in the combined group of *CC* and *CT* genotypes than in individuals with the *TT* genotype. BMD for the trochanter was greater in individuals with the *CT* genotype or in the combined group of *CC* and *CT* genotypes than in individuals with the *TT* genotype. The differences in BMD for D50, total body, and trochanter between the combined group of *CC* and *CT* geno-

SPANDIDOS PUBLICATIONS

. BMD and other characteristics for all women (n=1110) according to the *LIPC* genotype.[a]

Characteristic	*CC*	*CT*	*TT*	*CC + CT*	*CT + TT*
Number (%)	250 (22.5)	558 (50.3)	302 (27.2)	808 (72.8)	860 (77.5)
Age (years)	59.2±0.7	59.3±0.5	59.2±0.6	59.3±0.4	59.3±0.4
Height (cm)	151.2±0.4	151.3±0.3	151.4±0.3	151.3±0.2	151.4±0.2
Body weight (kg)	52.7±0.5	52.6±0.3	52.6±0.5	52.6±0.3	52.6±0.3
BMD measured with pQCT (mg/cm^3)					
D50	186.2±3.9	188.3±2.6	178.6±3.5	187.6±2.2[b]	184.8±2.1
D100	485.9±5.6	490.1±3.8	478.2±5.1	488.8±3.1	485.9±3.1
P100	1148.2±9.1	1159.1±6.1	1149.2±8.3	1155.7±5.1	1155.6±4.9
BMD measured with DXA (g/cm^2)					
Total body	0.956±0.005	0.974±0.004[c,d]	0.956±0.005	0.968±0.003[e]	0.968±0.003
L2-L4	0.859±0.008	0.873±0.005	0.856±0.007	0.869±0.004	0.867±0.004
Femoral neck	0.674±0.005	0.681±0.004	0.675±0.005	0.679±0.003	0.679±0.003
Trochanter	0.568±0.005	0.577±0.003[f]	0.562±0.005	0.574±0.003[g]	0.572±0.003

[a]BMD is adjusted for age, height, and body weight. Data are means ± SE. [b]P=0.0310, [d]P=0.0098, [e]P=0.0324, [f]P=0.0353, [g]P=0.0317 versus *TT*; [c]P=0.0176 versus *CC*.

Table X. BMD and other characteristics for postmenopausal women (n=815) according to the *LIPC* genotype.[a]

Characteristic	*CC*	*CT*	*TT*	*CC + CT*	*CT + TT*
Number (%)	181 (22.2)	406 (49.8)	228 (28.0)	587 (72.0)	634 (77.8)
Age (years)	64.2±0.6	64.1±0.4	63.3±0.6	64.1±0.4	63.8±0.3
Height (cm)	149.7±0.5	150.3±0.3	150.3±0.4	150.1±0.3	150.3±0.2
Body weight (kg)	52.2±0.6	51.7±0.4	52.4±0.5	51.8±0.4	52.0±0.3
BMD measured with pQCT (mg/cm^3)					
D50	164.7±4.7	167.6±3.2	156.1±4.2	166.7±2.6[b]	163.4±2.5
D100	442.3±6.8	447.7±4.6	436.2±6.0	446.1±3.8	443.6±3.6
P100	1073.7±11.3	1087.7±7.5	1071.3±10.0	1083.4±6.3	1081.8±6.0
BMD measured with DXA (g/cm^2)					
Total body	0.907±0.006	0.929±0.004[c,d]	0.911±0.006	0.922±0.004	0.922±0.003[e]
L2-L4	0.799±0.010	0.818±0.006	0.801±0.008	0.812±0.005	0.812±0.005
Femoral neck	0.639±0.006	0.649±0.004	0.641±0.006	0.646±0.003	0.646±0.003
Trochanter	0.537±0.006	0.545±0.004	0.533±0.005	0.543±0.003	0.541±0.003

[a]BMD is adjusted for age, height, and body weight. Data are means ± SE. [b]P=0.0334, [d]P=0.0348 versus *TT*; [c]P=0.0173, [e]P=0.0439 versus *CC*.

types and individuals with the *TT* genotype were 4.8, 1.2, and 2.1%, respectively.

For postmenopausal women, BMD for D50 was greater in the combined group of *CC* and *CT* genotypes than in individuals with the *TT* genotype (Table X). BMD for the total body was greater in individuals with the *CT* genotype than in those with the *CC* genotype or the *TT* genotype, and was greater in the combined group of *CT* and *TT* genotypes than in individuals with the *CC* genotype. The difference in BMD for D50 between the combined group of *CC* and *CT* genotypes and individuals with the *TT* genotype and that for the total body between the combined group of *CT* and *TT* genotypes and individuals with the *CC* genotype were 6.4 and 1.6%, respectively.

For premenopausal women or men, no relation was detected between *LIPC* genotype and BMD (data not shown).

Relation of the 1462A→G (Lys469Glu) polymorphism of ICAM1 to BMD. The distribution of 1462A→G genotypes of *ICAM1* was in Hardy-Weinberg equilibrium, and age and height did not differ among genotypes for all women (Table XI), premenopausal women, or postmenopausal women (Table XII). For all women and postmenopausal women, but not premenopausal women, body weight was greater in individuals with the *GG* genotype than in the combined group of *AA* and *AG* genotypes. For all women, BMD for D50 was greater in individuals with the *GG* genotype than in those with the *AA* genotype or in the combined group of *AA* and

Table XI. BMD and other characteristics for all women (n=1096) according to the *ICAM1* genotype.[a]

Characteristic	AA	AG	GG	AA + AG	AG + GG
Number (%)	364 (33.2)	536 (48.9)	196 (17.9)	900 (82.1)	732 (66.8)
Age (years)	60.0±0.6	58.9±0.5	59.1±0.8	59.3±0.4	59.0±0.4
Height (cm)	151.1±0.3	151.3±0.3	151.7±0.4	151.2±0.2	151.4±0.2
Body weight (kg)	52.3±0.4	52.4±0.4	53.9±0.6	52.3±0.3[b]	52.8±0.3
BMD measured with pQCT (mg/cm^3)					
D50	177.6±3.2[c]	186.6±2.7	194.9±4.4	182.9±2.1[d]	188.8±2.3[e]
D100	481.8±4.6[f]	483.1±3.9[g]	500.8±6.4	482.6±3.0[h]	487.9±3.3
P100	1143.5±7.5	1154.3±6.2	1169.9±10.3	1149.9±4.8	1158.5±5.3
BMD measured with DXA (g/cm^2)					
Total body	0.960±0.004	0.965±0.004	0.973±0.006	0.963±0.003	0.967±0.003
L2-L4	0.856±0.007	0.870±0.006	0.869±0.009	0.864±0.004	0.870±0.005
Femoral neck	0.677±0.004	0.677±0.004	0.679±0.006	0.677±0.003	0.678±0.003
Trochanter	0.564±0.004	0.574±0.004	0.576±0.006	0.570±0.003	0.574±0.003

[a]BMD is adjusted for age, height, and body weight. Data are means ± SE. [b]P=0.0148, [c]P=0.0046, [d]P=0.0142, [f]P=0.0439, [g]P=0.0489, [h]P=0.0101 versus *GG*; [e]P=0.0046 versus *AA*.

Table XII. BMD and other characteristics for postmenopausal women (n=807) according to the *ICAM1* genotype.[a]

Characteristic	*AA*	*AG*	*GG*	*AA + AG*	A*G + GG*
Number (%)	274 (34.0)	386 (47.8)	147 (18.2)	660 (81.8)	533 (66.0)
Age (years)	64.3±0.5	63.9±0.4	63.1±0.7	64.1±0.3	63.7±0.4
Height (cm)	150.0±0.4	150.0±0.3	150.8±0.5	150.0±0.2	150.2±0.3
Body weight (kg)	51.6±0.5	51.7±0.4	53.5±0.7	51.7±0.3[b]	52.2±0.4
BMD measured with pQCT (mg/cm^3)					
D50	156.3±3.8[c]	163.9±3.3	176.0±5.2	160.7±2.5[d]	167.3±2.8[e]
D100	438.1±5.5	440.3±4.7	459.4±7.5	439.3±3.6[f]	445.6±4.0
P100	1068.4±9.1	1080.4±7.8	1097.6±12.5	1075.3±5.9	1085.2±6.6
BMD measured with DXA (g/cm^2)					
Total body	0.910±0.005[g]	0.919±0.004	0.936±0.007	0.915±0.003[h]	0.923±0.004[i]
L2-L4	0.796±0.008	0.813±0.007	0.823±0.011	0.806±0.005	0.816±0.006[j]
Femoral neck	0.639±0.005	0.645±0.004	0.652±0.007	0.643±0.003	0.647±0.004
Trochanter	0.531±0.005[k]	0.543±0.004	0.551±0.007	0.538±0.003	0.545±0.004[l]

[a]BMD is adjusted for age, height, and body weight. Data are means ± SE. [b]P=0.0147, [c]P=0.0068, [d]P=0.0083, [f]P=0.0166, [g]P=0.0073, [h]P=0.0066, [k]P=0.0436 versus *GG*; [e]P=0.0205, [i]P=0.0319, [j]P=0.0441, [l]P=0.0220 versus *AA*.

AG genotypes, and was greater in the combined group of *AG* and *GG* genotypes than in individuals with the *AA* genotype (Table XI). BMD for D100 was greater in individuals with the *GG* genotype than in those with the *AA* genotype or the *AG* genotype or in the combined group of *AA* and *AG* genotypes. The differences in BMD for D50 and D100 between individuals with the *GG* genotype and those with the *AA* genotype were 8.9 and 3.8%, respectively.

For postmenopausal women, BMD for D50 and that for the total body were greater in individuals with the *GG* genotype than in those with the *AA* genotype or in the combined group of *AA* and *AG* genotypes, and were greater in the combined group of *AG* and *GG* genotypes than in individuals with the *AA* genotype (Table XII). BMD for D100 was greater in individuals with the *GG* genotype than in the combined group of *AA* and *AG* genotypes. BMD for the lumbar spine was greater in the combined group of *AG* and *GG* genotypes than in individuals with the *AA* genotype. BMD for the trochanter was greater in individuals with the *GG* genotype or in the combined group of *AG* and *GG* genotypes than in individuals with the *AA* genotype. The differences in BMD for D50, total body, and trochanter between individuals with the *GG* genotype and those with the *AA* genotype were 11.2, 2.8, and 3.6%, respectively. The difference in BMD for D100

SPANDIDOS PUBLICATIONS

individuals with the *GG* genotype and the combined *AA* and *AG* genotypes and that for the lumbar spine between the combined group of *AG* and *GG* genotypes and individuals with the *AA* genotype were 4.4 and 2.5%, respectively. For premenopausal women, no relation was detected between *ICAM1* genotype and BMD.

For men, the distribution of A→G genotypes of *ICAM1* was in Hardy-Weinberg equilibrium, and BMD for P100 was greater in the combined group of *AA* and *AG* genotypes than in individuals with the *GG* genotype (data not shown).

Discussion

We have examined the relations of six candidate gene polymorphisms to BMD at various sites in community-dwelling Japanese women and men. Our results showed that the polymorphisms of *ALAP* and *PLOD1* were associated with BMD in premenopausal women; those of *ICAM1* and *LIPC* with BMD in postmenopausal women; that of *CNR2* with BMD in premenopausal and postmenopausal women; and that of *GJA4* with BMD in men. Among these polymorphisms, those of *ICAM1*, *CNR2*, and *GJA4* were markedly associated with BMD. These observations thus suggest that *ALAP*, *PLOD1*, I*CAM1*, *LIPC*, and *CNR2* are susceptibility loci for reduced bone mass in Japanese women and that *GJA4* constitutes such a locus in Japanese men. The polymorphisms of *ICAM1* and *CNR2* may confer susceptibility to postmenopausal osteoporosis in women, whereas that of *GJA4* may confer susceptibility to osteoporosis in men.

Association of the 386G→A (Ala99Thr) polymorphism of PLOD1 with BMD. *PLOD1* is located within a quantitative trait locus for regulation of BMD on chromosome 1p36 (17) and is a strong candidate gene for the regulation of BMD. *PLOD1* encodes the enzyme procollagen-lysine, 2-oxoglutarate 5-dioxygenase, which catalyzes the hydroxylation of lysine residues during the posttranslational modification of type I collagen, the major protein of bone matrix. Tasker *et al* (18) detected an association between BMD for the lumbar spine and the 386G→A (Ala99Thr) polymorphism of *PLOD1* in a population-based cohort of 678 Scottish women. Heterozygotes for this polymorphism had a reduced BMD and an increased hydroxylysylpyridinoline to lysylpyridinoline ratio compared with either group of homozygotes, suggesting a functional effect of this polymorphism on enzyme activity. Spotila *et al* (19) obtained evidence for an allelic association between a T→G polymorphism in intron 6 of *PLOD1* and BMD for the lumbar spine. This polymorphism and the 386G→A (Ala99Thr) polymorphism were in complete linkage disequilibrium. We have now shown that the 386G→A (Ala99Thr) polymorphism of *PLOD1* was associated with BMD for the femoral neck and trochanter for all women and premenopausal women, with the *A* allele being associated with reduced BMD. Our present results and the previous observations (18,19) thus suggest that *PLOD1* may be a susceptibility gene for reduced BMD in women.

Association of the A→G polymorphism of CNR2 with BMD. Two cannabinoid receptors, CB1 and CB2, encoded by *CNR1* and *CNR2*, respectively, are highly homologous, belong to the family of G protein-coupled seven-transmembrane domain receptors, and bind and are activated by endocannabinoids. *CNR1* is expressed predominantly in the brain and peripheral neurons (20), whereas *CNR2* is expressed mainly in immune cells (21). Mice with a targeted deletion of *CNR1* have an increased bone mass (22), whereas *CNR2* knockout mice have a decreased bone mass resembling human osteoporosis (23). These mouse genetic data implicate the endocannabinoid system in the regulation of bone mass. Furthermore, given that *CNR2* is located at chromosomal region 1p36, which has been implicated in osteoporosis (17), *CNR2* is a strong candidate determinant of susceptibility to osteoporosis. Karsak *et al* (24) detected an association of single polymorphisms and haplotypes encompassing *CNR2* on chromosome 1p36 with osteoporosis. We have now shown that the A→G polymorphism of *CNR2* was associated with BMD for the distal radius, total body, and lumbar spine in all women, with BMD for the distal radius and lumbar spine in premenopausal women, and with BMD for the distal radius, total body, and trochanter in postmenopausal women, with the *G* allele being related to reduced BMD. Our present results and the previous association study (24), as well as the observations with CB2-deficient mice (23), thus suggest that *CNR2* is a susceptibility gene for reduced BMD in women.

Association of the 1019C→T (Pro319Ser) polymorphism of GJA4 with BMD. Gap junction protein, α-4 (connexin37) is a gap junction protein in the arterial endothelium and contributes to the growth and regeneration after injury of endothelial cells (25). It forms functional intercellular channels with a voltage dependence and unitary conductance properties that are distinct from those of other channels (26). The 1019C→T (Pro319Ser) polymorphism of *GJA4* was previously associated with myocardial infarction (16) and coronary heart disease (27), with the *T* allele representing a risk factor for these conditions. Wong *et al* (28) recently showed that GJA4 protects against excessive monocyte recruitment in atherosclerosis, revealing an anti-inflammatory role for this protein *in vivo*. These researchers also showed that mononuclear cells expressing the 1019T (Ser319) allele of the *GJA4* polymorphism exhibited stronger adhesion than those expressing the 1019C (Pro319) allele, consistent with the observations that the 1019T (Ser319) allele is associated with an increased risk of myocardial infarction (16) and coronary heart disease (27). The anti-adhesive effect of GJA4 was shown to be mediated by release of ATP into the extracellular space. GJA4 hemichannels may thus control initiation of the development of atherosclerotic plaques by regulating monocyte adhesion (28). We have now shown that the 1019C→T (Pro319Ser) polymorphism of *GJA4* was associated with BMD for the total body, lumbar spine, femoral neck, and trochanter in men, with the *T* allele being associated with reduced BMD. This is the first demonstration of an association of *GJA4* with BMD, although the underlying molecular mechanism of the effect of this polymorphism on bone remodeling remains to be elucidated.

Association of the 1583G→A (Arg528Lys) polymorphism of ALAP with BMD. Adipocyte-derived leucine aminopeptidase

(ALAP) has been identified as a member of the M1 family of zinc-dependent metallopeptidases (29). ALAP was shown to catalyze the hydrolysis of a variety of bioactive peptides *in vitro* and to play a role in the regulation of blood pressure through inactivation of angiotensin II and generation of bradykinin (29). The 1583G→A (Arg528Lys) polymorphism of *ALAP* was associated with essential hypertension, with the 1583G (Arg528) allele representing a risk factor for this condition (30). We have now shown that the 1583G→A (Arg528Lys) polymorphism of *ALAP* was associated with BMD for the total body, femoral neck, and trochanter in premenopausal women, with the *A* allele being associated with reduced BMD. The molecular mechanism responsible for the effect of this polymorphism on bone remodeling remains to be determined.

Association of the -514C→T polymorphism of LIPC with BMD. Hepatic lipase (LIPC), a glycoprotein member of the lipase superfamily, plays an important role in the metabolism and modeling of both pro- and anti-atherogenic lipoproteins. Synthesized and secreted by the liver, LIPC performs several metabolic functions, including hydrolysis of triglycerides and phospholipids, modeling of certain low-density lipoprotein (LDL)-cholesterol particles, and catabolism of high-density lipoprotein (HDL) cholesterol (31). The -514C→T polymorphism of *LIPC*, which is located in the promoter region, has been shown to affect LIPC activity, with activity being decreased in carriers of the *T* allele (32,33). This polymorphism was also shown to have a significant effect on the plasma level of HDL cholesterol, with the *T* allele being associated with increased levels (34,35). A meta-analysis of 25 studies, including a total of >24,000 subjects, revealed significant decreases in plasma LIPC activity and increases in HDL-cholesterol levels in individuals with the *CT* and those with the *TT* genotype compared with those with the *CC* genotype of the -514C→T polymorphism (36). We have now shown that this polymorphism was associated with BMD for the distal radius, total body, and trochanter in all women and with BMD for the distal radius and total body in postmenopausal women, with the *CT* genotype being associated with increased BMD. The mechanisms responsible for the associations of the *CT* genotype both with increased plasma concentrations of HDL (36) and with increased bone mass in women (the present study) remain to be elucidated.

Association of the 1462A→G (Lys469Glu) polymorphism of ICAM1 with BMD. Interactions between osteoblasts and osteoclasts are important in osteoclastogenesis, and multiple adhesion molecules, including intercellular adhesion molecule 1 (ICAM1 or CD54) (37,38), are expressed on the osteoblast surface. Studies of osteoclastogenesis in coculture systems of osteoblasts and preosteoclastic cells have revealed that inhibition of the cellular interactions mediated through ICAM1 with the use of specific monoclonal antibodies inhibited osteoclast formation (38,39). These studies thus demonstrated a pivotal role for ICAM1-expressing osteoblasts in the differentiation of osteoclast precursor cells into mature osteoclasts, resulting in a shift in bone homeostasis toward resorption. Indeed, the expression of *ICAM1* in osteoblasts was shown to be increased in osteoporotic bone (40). The 1462A→G (Lys469Glu) polymorphism of *ICAM1*, which is located in a region of the gene corresponding to an immunodominant epitope involved in integrin-mediated B cell adhesion and neutrophil transmigration, has been associated with a variety of proinflammatory phenotypes including transplant rejection and vasculopathy (41) as well as postoperative myocardial infarction (42). We have now shown that this polymorphism of *ICAM1* was associated with BMD for the distal radius in all women, and with BMD for the distal radius, total body, lumbar spine, and trochanter in postmenopausal women, with the *A* allele being associated with reduced BMD. The association of the 1462A→G (Lys469Glu) polymorphism with BMD may be attributed to the effect of this polymorphism on osteoclastogenesis and consequent bone resorption.

Limitations of the study. Given the multiple comparisons of genotypes with BMD at various sites in the present study, it is not possible to exclude potential type I errors (false positives). It is also possible that the polymorphisms associated with reduced BMD in our study were in linkage disequilibrium with other polymorphisms in the same gene or polymorphisms of nearby genes that are actually responsible for the development of this condition. Furthermore, the relevance of the polymorphisms to gene transcription or to protein structure or function and their effects on bone remodeling were not determined in the present study.

In conclusion, our present results suggest that *ALAP*, *PLOD1*, *ICAM1*, *LIPC*, and *CNR2* are susceptibility loci for reduced BMD in Japanese women and that *GJA4* constitutes such a locus in Japanese men. The polymorphisms of *ICAM1* and *CNR2* may confer susceptibility to postmenopausal osteoporosis in women, and that of *GJA4* to osteoporosis in men. Determination of genotypes for these polymorphisms may prove informative for assessment of the genetic risk for reduced BMD. Given that multiple variants, each having a small effect, will likely ultimately be found to be responsible for a large fraction of the genetic component of osteoporosis, identification of additional osteoporosis susceptibility genes will allow more accurate assessment of the genetic component of this condition.

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