

Association of gene polymorphisms with blood pressure and the prevalence of hypertension in community-dwelling Japanese individuals

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Abstract. Hypertension is a complex multifactorial disorder that is thought to result from an interaction between genetic background and environmental factors. Although various loci and genes have been implicated in predisposition to hypertension by genetic linkage analyses and candidate gene association studies, the genes that confer susceptibility to this condition remain to be identified definitively. We examined the relations of nine candidate gene polymorphisms to blood pressure (BP) and the prevalence of hypertension in a population-based study. The 2238 subjects (1110 women, 1128 men) were aged 40 to 79 years and were randomly recruited for a population-based prospective cohort study of aging and age-related diseases in Japan. BP was measured with subjects having rested in a sitting position for at least 15 min. Genotypes for the 160C→T (Arg54Trp) polymorphism of *QPCT*, the C→T (Pro198Leu) polymorphism of *GPX1*, the 137,346T→C polymorphism of *FYN*, the -344C→T polymorphism of *CYP11B2*, and the A→G (Ser49Gly) polymorphism of *ADRB1* were determined with a fluorescence-based allele-specific DNA primer assay system; those for the A→G polymorphism of *CNR2*, the 1D (22,375delAC) polymorphism of *CAVI*, and the -1213T→C polymorphism of *ESR2* by melting curve analysis, and that for the (GT)_n polymorphism of *COL1A2* were determined by DNA fragment analysis. The polymorphism of *FYN* was associated with systolic and diastolic BP in women. In men, polymorphisms of *CNR2*, *QPCT*, *GPX1*, *COL1A2*, *CYP11B2*, and *ESR2* were associated with systolic and diastolic BP, those of *CAVI* and *FYN* with systolic BP, and that of *ADRB1* with diastolic BP. The polymorphisms of *QPCT* and *CYP11B2* were also

associated with the prevalence of hypertension in men. These results suggest that polymorphisms of *QPCT* and *CYP11B2* are determinants of BP and the development of hypertension in Japanese men.

Introduction

Hypertension is a complex multifactorial disorder that is thought to result from an interaction between an individual's genetic background and various environmental factors (1). Given that hypertension is a major risk factor for coronary heart disease, stroke, and chronic renal failure, personalized prevention of hypertension is an important public health goal. One approach to personalized prevention of, and selection of the most appropriate treatment for, hypertension is to identify genes that confer susceptibility to this condition. Although genetic linkage analyses (2-5) and candidate gene association studies (6-9) have implicated various loci and genes in the predisposition to hypertension, the genes that confer genetic susceptibility to this condition remain to be identified definitively. In addition, because of ethnic divergence of gene polymorphisms as well as of environmental factors and lifestyle, it is important to examine polymorphisms related to hypertension in each ethnic group.

We have been attempting to identify genes significantly associated with blood pressure (BP) in Japanese women or men with a population-based approach. In the present study, we selected nine candidate genes that might be expected to contribute to the regulation of BP (Table I) and examined the relations of polymorphisms of these genes to BP, even though there was no apparent biological link among these genes. Our aim was to identify a single polymorphism significantly associated with BP 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 BP and the prevalence of hypertension in community-dwelling Japanese women and men.

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Materials and methods

Study population. The National Institute for Longevity Sciences, the Longitudinal Study of Aging, is a population-

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

Locus	Gene	Symbol	Polymorphism	dbSNP
1p36.11	Cannabinoid receptor 2	<i>CNR2</i>	A-G	rs2501431
2p22.2	Glutaminyl-peptide cyclotransferase	<i>QPCT</i>	160C-T (Arg54Trp)	rs2255991
3p21.3	Glutathione peroxidase	<i>GPX1</i>	C-T (Pro198Leu)	rs1050450
6q21	FYN oncogene related to SRC, FGR, YES	<i>FYN</i>	137,346T-C	rs706895
7q22.1	Collagen, type I, α -2	<i>COL1A2</i>	(GT) _n	ND
7q31.1	Caveolin 1	<i>CAV1</i>	1D (22,375delAC)	rs3840634
8q21-q22	Cytochrome P450, subfamily Y XIB, polypeptide 2	<i>CYP11B2</i>	-344C-T	rs1799998
10q24-q26	β -1-adrenergic receptor	<i>ADRB1</i>	A-G (Ser49Gly)	rs1801252
14q23.2	Estrogen receptor 2	<i>ESR2</i>	-1213T-C	ND

ND, not detected in dbSNP.

based prospective cohort study of aging and age-related diseases (10). The subjects were unrelated individuals stratified by both age and sex, and were randomly selected from resident registrations in the city of Obu and town of Higashiura in central Japan (11-13). The lifestyle of residents of this area is typical of that of individuals in most regions of Japan. The numbers of men and women recruited were similar and the age at baseline was 40-79 years, with similar numbers of participants in each decade (40, 50, 60 and 70s). The subjects are being followed up every 2 years. All participants were subjected at a special center to a detailed examination, which included not only medical evaluation but also assessment of exercise physiology, body composition, nutrition, and psychology. Individuals with coronary heart disease, valvular heart disease, cardiomyopathies, or renal or endocrinologic diseases that cause secondary hypertension were excluded from the present study. We thus examined the relations of gene polymorphisms to BP or the prevalence of hypertension in 2238 individuals (1110 women, 1128 men). Individuals whose genotypes were not successfully determined were excluded from the analysis. The study protocol complies 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 BP. BP was measured with an automatic sphygmomanometer (BP-203RV-II; Colin, Tokyo, Japan) in subjects having rested in a sitting position for at least 15 min. BP in each subject was confirmed with the measurement made by a physician with a mercury manometer according to the guidelines of the American Heart Association (14). Normal BP was defined as both a systolic BP of <140 mmHg and a diastolic BP of <90 mmHg. Hypertension was defined as a systolic BP of \geq 140 mmHg or a diastolic BP of \geq 90 mmHg (or both), or the use of antihypertensive medication.

Determination of genotype. Genotypes for polymorphisms of *QPCT*, *GPX1*, *FYN*, *CYP11B2*, and *ADRB1* were determined with a fluorescence-based allele-specific DNA primer assay system (Toyobo Gene Analysis, Tsuruga, Japan) (15). 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 or Texas red and with an antisense primer labeled at the 5' end with biotin. The reaction mixture (25 μ l) contained 20 ng of DNA, 5 pmol of each primer, 0.2 mmol/l of each deoxynucleoside triphosphate, 2.5-4 mmol/l MgCl₂, and 1 U of rTaq DNA polymerase (Toyobo, Osaka, Japan) in polymerase buffer. The amplification protocol comprised initial denaturation at 95°C for 5 min; 35 cycles of denaturation at 95°C for 30 sec, annealing at 60-70°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 fluorescein isothiocyanate and of 584 and 612 nm, respectively, for Texas red.

Genotypes for polymorphisms of *CNR2*, *CAV1*, and *ESR2* 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 μ l) containing 20 ng of DNA, 5 pmol of each primer, 0.2 mmol/l of each deoxynucleoside triphosphate, 2 mmol/l MgCl₂, and 1.25 U of rTaq DNA polymerase in polymerase buffer. The amplification protocol comprised initial denaturation at 95°C for 5 min; 45 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 μ l) 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) 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.

Table II. Primers, probes, and other PCR conditions for genotyping.

Gene	Polymorphism	Sense primer with FITC	Sense primer with Texas red			
<i>QPCT</i>	160C-T (Arg54Trp)	TTTGAATTCATCGGCTCTxCG	TTTGAATTCATCGGCTCTxTG			
<i>GPXI</i>	C-T (Pro198Leu)	GCGCCCTAGGCACAGCTxAG	GCGCCCTAGGCACAGCTxGG			
<i>FYN</i>	137,346T-C	GGAGTAATTGACAAGGCTCAxCG	AGGAGTAATTGACAAGGCTCAxTG			
<i>CYP11B2</i>	-344C-T	TATTAAGAATCCAAGGxCC	GTCTATTAAGAATCCAAGGxTC			
<i>ADRB1</i>	A-G (Ser49Gly)	GAGACAGCGGCTCGGGGxCT	GACAGCGGCTCGGGGxTT			
		Antisense primer with biotin	Annealing (°C)	Cycles	Mg ²⁺ (mM)	Taq/KOD
<i>QPCT</i>		GGTATCGCTCTATCAGCAATGG	62.5	35	2.5	Taq
<i>GPXI</i>		GTGTGCCCTACGCAGGTACA	65.0	35	2.5	Taq
<i>FYN</i>		CCTTTCCTCATGCCCCCTAAT	67.5	35	4.0	Taq
<i>CYP11B2</i>		GGACTTTATCTTATCGTGAGATGA	60.0	35	3.0	Taq
<i>ADRB1</i>		GCCGCCCGCCTCGTTG	70.0	35	3.5	Taq
Gene	Polymorphism	Sense primer	Antisense primer			
<i>CNR2</i>	A-G	GGGCAGGTAGGAGACTAGTGCTGAGAG	CTCACCCGTGGAAGGGCACTG			
<i>CAVI</i>	I/D (22,375delAC)	AAAGGTGATGGATCATTTCCTATTATACAC	TGGGCAATGGTCATCCATGACTG			
<i>ESR2</i>	-1213T-C	GAACAGGAGCCAGGGGCACAG	CCTGAAGACAAGTACCTTGCAGCTGAG			
		Probe	Annealing (°C)	Cycles	Mg ²⁺ (mM)	Taq/KOD
<i>CNR2</i>		CACATGATGCCAGGGTC	65.0	45	2.0	Taq
<i>CAVI</i>		CAAAATGTGTGCCATTTCAGG	65.0	45	2.0	Taq
<i>ESR2</i>		AACAGTAAAATTCTGCCTGGG	65.0	45	2.0	Taq
Gene	Polymorphism	Sense primer with FAM	Antisense primer			
<i>COLIA2</i>	(GT) _n	CAGCACGGTGTCTACCACTGC	ATTACTCCTTAGTATCCACAGTATGTATAC			
		Annealing (°C)	Cycles	Mg ²⁺ (mM)	Taq/KOD	
<i>COLIA2</i>		60.0	35	1.2	KOD	

FITC, fluorescein isothiocyanate; FAM, 6-carboxyfluorescein. Oligonucleotide sequences are 5'-3'.

The GT repeats [(GT)_n] in the first intron of *COLIA2* were amplified by PCR with a sense primer labeled at the 5' end with 6-carboxyfluorescein and with an antisense primer (Table II). The reaction mixture (25 µl) contained 20 ng of DNA, 5 pmol of each primer, 0.2 mmol/l of each deoxynucleoside triphosphate, 1.2 mmol/l MgSO₄, and 0.4 U of KOD plus DNA polymerase (Toyobo) in polymerase buffer. The amplification protocol comprised initial denaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 15 sec, annealing at 60°C for 30 sec, and extension at 68°C for 30 sec; and a final extension at 68°C for 2 min. The size of the (GT)_n-containing PCR products was determined with a PRISM 3100 DNA sequencer and with GeneScan and Genotyper software (Applied Biosystems).

Statistical analysis. Age and BP were compared among three groups by one-way analysis of variance and the Tukey-Kramer *post hoc* test, and between two groups by the unpaired Student's *t*-test. RP values were analyzed in

individuals who were not taking antihypertensive drugs. The prevalence of hypertension was compared between two groups (2x2) or among three groups (3x2) by the Chi-square test in all individuals. Allele frequencies were estimated by the gene-counting 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 A-G polymorphism of *CNR2* to BP. For men, the distribution of genotypes for the A-G polymorphism of *CNR2* was in Hardy-Weinberg equilibrium and individuals in the combined group of AG and GG genotypes were younger than those with the AA genotype (Table III). Systolic and diastolic BP were significantly higher in men with the GG genotype than in those with the AA genotype or with the AG genotype or in those in the combined group of AA and AG

Table III. Blood pressure (BP) for male subjects according to *CNR2* genotype.

Characteristics	AA	AG	GG	AA + AG	AG + GG
Number of subjects (n=874) ^a	295	425	154	720	579
Age (years)	60.2±0.6	58.5±0.5	59.2±0.8	59.2±0.4	58.7±0.4 ^b
Systolic BP (mmHg)	120.5±1.0 ^c	120.3±0.8 ^d	125.0±1.4	120.4±0.6 ^e	121.6±0.7
Diastolic BP (mmHg)	74.8±0.6 ^f	74.9±0.5 ^g	78.0±0.9	74.9±0.4 ^h	75.8±0.4
Number of all subjects (n=1122)	387	549	186	936	735
Hypertension (%)	34.4	33.5	37.6	33.9	34.6

^aSubjects not taking antihypertensive medication. Data for age and BP are means ± SE. Data for the combined group of AA and AG genotypes (AA + AG) were compared with those for individuals with the GG genotype (recessive genetic model). Data for the combined group of AG and GG genotypes (AG + GG) were compared with those for individuals with the AA genotype (dominant genetic model). ^bP=0.0313 versus AA; ^cP=0.0230, ^dP=0.0101, ^eP=0.0025, ^fP=0.0071, ^gP=0.0065, ^hP=0.0010 versus GG.

Table IV. Blood pressure (BP) for male subjects according to *QPCT* genotype.

Characteristics	CC	CT	TT	CC + CT	CT + TT
Number of subjects (n=878) ^a	387	379	112	766	491
Age (years)	58.8±0.5	59.8±0.5	58.4±0.9	59.3±0.4	59.5±0.4
Systolic BP (mmHg)	119.5±0.9	122.4±0.9	121.7±1.7	121.1±0.6	122.3±0.8 ^b
Diastolic BP (mmHg)	74.6±0.5	76.3±0.5	75.1±1.0	75.5±0.4	76.1±0.5 ^c
Number of all subjects (n=1128)	487	501	140	988	641
Hypertension (%)	29.2 ^e	39.5 ^e	35.0 ^e	34.4	38.5 ^d

^aSubjects not taking antihypertensive medication. Data for age and BP are means ± SE. Data for the combined group of CC and CT genotypes (CC + CT) were compared with those for individuals with the TT genotype (recessive genetic model). Data for the combined group of CT and TT genotypes (CT + TT) were compared with those for individuals with the CC genotype (dominant genetic model). ^bP=0.0233, ^cP=0.0434, ^dP=0.0010 versus CC. ^eP=0.0027 (CC versus CT versus TT, 3x2 Chi-square test).

genotypes. The differences in systolic and diastolic BP between individuals with the GG genotype and those in the combined group of AA and AG genotypes (expressed as a percentage of the larger value) were 3.7 and 4.0%, respectively. The prevalence of hypertension did not differ among *CNR2* genotypes for men. For women, neither systolic or diastolic BP nor the prevalence of hypertension differed among *CNR2* genotypes (data not shown).

Relation of the 160C→T (Arg54Trp) polymorphism of QPCT to BP. The distribution of genotypes for the 160C→T polymorphism of *QPCT* was in Hardy-Weinberg equilibrium and age did not differ among genotypes for men (Table IV). Systolic and diastolic BP were significantly greater for men in the combined group of CT and TT genotypes than for those with the CC genotype; the differences in systolic and diastolic BP between these groups were 2.3 and 2.0%, respectively. The prevalence of hypertension also differed significantly among genotypes (CC versus CT versus TT), being greater for men in the combined group of CT and TT genotypes than for those with the CC genotype. The odds ratio of the T allele compared with the C allele for predisposition to hypertension was 1.3. There were no differences in systolic or diastolic BP or in the prevalence of hypertension among *QPCT* genotypes in women (data not shown).

Relation of the C→T (Pro198Leu) polymorphism of GPXI to BP. Among men, the distribution of genotypes for the C→T (Pro198Leu) polymorphism of *GPXI* was in Hardy-Weinberg equilibrium and age did not differ among genotypes (Table V). Systolic and diastolic BP were significantly higher for men with the CT genotype or for those in the combined group of CT and TT genotypes than for those with the CC genotype. The differences in systolic and diastolic BP between individuals in the combined group of CT and TT genotypes and those with the CC genotype were 3.6 and 3.7%, respectively. The prevalence of hypertension did not differ among *GPXI* genotypes for men. Among women, no difference in systolic or diastolic BP or in the prevalence of hypertension was detected among *GPXI* genotypes (data not shown).

Relation of the 137,346T→C polymorphism of FYN to BP. For women, the distribution of genotypes for the 137,346T→C polymorphism of *FYN* was in Hardy-Weinberg equilibrium and age did not differ among genotypes (Table VI). Systolic and diastolic BP were significantly higher in women with the TC genotype or in those in the combined group of TC and CC genotypes than in those with the TT genotype. The differences in systolic and diastolic BP between individuals in the combined group of TC and CC genotypes and those

Table V. Blood pressure (BP) for male subjects according to *GPX1* genotype.

Characteristics	CC	CT	TT	CC + CT	CT + TT
Number of subjects (n=879) ^a	750	126	3	876	129
Age (years)	59.3±0.4	58.7±0.9	59.0±6.3	59.2±0.4	58.7±0.9
Systolic BP (mmHg)	120.5±0.6	125.0±1.5 ^b	122.0±9.9	121.2±0.6	125.0±1.5 ^c
Diastolic BP (mmHg)	75.0±0.4	78.0±0.9 ^d	71.7±6.1	75.4±0.4	77.9±0.9 ^e
Number of all subjects (n=1128)	971	154	3	1125	157
Hypertension (%)	34.0	37.7	0	34.5	36.9

^aSubjects not taking antihypertensive medication. Data for age and BP are means ± SE. Data for the combined group of CC and CT genotypes (CC + CT) were compared with those for individuals with the TT genotype (recessive genetic model). Data for the combined group of CT and TT genotypes (CT + TT) were compared with those for individuals with the CC genotype (dominant genetic model). ^bP=0.0188, ^cP=0.0072, ^dP=0.0078, ^eP=0.0040 versus CC.

Table VI. Blood pressure (BP) for female subjects according to *FYN* genotype.

Characteristics	TT	TC	CC	TT + TC	TC + CC
Number of subjects (n=883) ^a	338	423	122	761	545
Age (years)	59.0±0.5	59.6±0.5	58.1±0.9	59.3±0.4	59.3±0.3
Systolic BP (mmHg)	117.9±1.0	121.6±0.9 ^b	119.6±1.7	119.9±0.7	121.1±0.8 ^c
Diastolic BP (mmHg)	71.5±0.6	73.8±0.5 ^d	72.4±1.0	72.8±0.4	73.5±0.5 ^e
Number of all subjects (n=1109)	426	532	151	958	683
Hypertension (%)	32.4	37.3	31.8	35.1	36.1

^aSubjects not taking antihypertensive medication. Data for age and BP are means ± SE. Data for the combined group of TT and TC genotypes (TT + TC) were compared with those for individuals with the CC genotype (recessive genetic model). Data for the combined group of TC and CC genotypes (TC + CC) were compared with those for individuals with the TT genotype (dominant genetic model). ^bP=0.0217, ^cP=0.0138, ^dP=0.0132, ^eP=0.0104 versus TT.

Table VII. Blood pressure (BP) for male subjects according to *FYN* genotype.

Characteristics	TT	TC	CC	TT + TC	TC + CC
Number of subjects (n=875) ^a	339	409	127	748	536
Age (years)	59.0±0.5	59.4±0.5	59.1±0.9	59.2±0.4	59.4±0.4
Systolic BP (mmHg)	119.5±1.0	122.3±0.9	121.3±1.6	121.2±0.6	122.1±0.7 ^b
Diastolic BP (mmHg)	74.7±0.6	75.8±0.5	76.1±0.9	75.3±0.4	75.9±0.5
Number of all subjects (n=1122)	441	527	154	968	681
Hypertension (%)	32.4	36.6	31.8	34.7	35.5

^aSubjects not taking antihypertensive medication. Data for age and BP are means ± SE. Data for the combined group of TT and TC genotypes (TT + TC) were compared with those for individuals with the CC genotype (recessive genetic model). Data for the combined group of TC and CC genotypes (TC + CC) were compared with those for individuals with the TT genotype (dominant genetic model). ^bP=0.0344 versus TT.

with the TT genotype were 2.6 and 2.7%, respectively. The prevalence of hypertension did not differ among *FYN* genotypes for women.

For men, the distribution of *FYN* genotypes was in Hardy-Weinberg equilibrium and age did not differ among genotypes (Table VII). Systolic BP was significantly higher for men in the combined group of TC and CC genotypes than for those with the TT genotype; the difference in systolic BP

between these groups was 2.1%. The prevalence of hypertension did not differ among *FYN* genotypes for men.

Relation of the (GT)_n polymorphism of COLIA2 to BP. Given that the mean and median numbers of GT repeats for *COLIA2* in the study subjects were 14.2 and 12, respectively, we designated (GT)_{n≤12} and (GT)_{n≥13} as short (S) and long (L) alleles, respectively. The distribution of the SS, SL, and LL

Table VIII. Blood pressure (BP) for male subjects according to *COL1A2* genotype.

Characteristics	SS	SL	LL	SS + SL	SL + LL
Number of subjects (n=854) ^a	252	415	187	667	602
Age (years)	59.7±0.6	59.4±0.5	58.7±0.7	59.5±0.4	59.2±0.4
Systolic BP (mmHg)	123.1±1.1	120.4±0.9	120.6±1.3	121.4±0.7	120.5±0.7 ^b
Diastolic BP (mmHg)	76.8±0.7	74.7±0.5 ^c	75.4±0.8	75.5±0.4	74.9±0.4 ^d
Number of all subjects (n=1095)	308	543	244	851	787
Hypertension (%)	33.3	34.6	36.1	34.2	35.1

^aSubjects not taking antihypertensive medication. Data for age and BP are means ± SE. S, short repeat allele [(GT)_n ≤12]; L, long repeat allele [(GT)_n ≥13]. Data for the combined group of SS and SL genotypes (SS + SL) were compared with those for individuals with the LL genotype (recessive genetic model). Data for the combined group of SL and LL genotypes (SL + LL) were compared with those for individuals with the SS genotype (dominant genetic model). ^bP=0.0460, ^cP=0.0341, ^dP=0.0178 versus SS.

Table IX. Blood pressure (BP) for male subjects according to *CAVI* genotype.

Characteristics	II	ID	DD	II + ID	ID + DD
Number of subjects (n=879) ^a	796	82	1	878	83
Age (years)	59.1±0.3	60.6±1.1	48.0	59.2±0.4	60.4±1.1
Systolic BP (mmHg)	120.8±0.6	125.0±1.9	126.0	121.2±0.6	125.1±1.9 ^b
Diastolic BP (mmHg)	75.2±0.4	77.4±1.2	75.0	75.4±0.4	77.3±1.2
Number of all subjects (n=1128)	1028	99	1	1127	100
Hypertension (%)	34.2	36.4	0	34.4	36.0

^aSubjects not taking antihypertensive medication. Data for age and BP are means ± SE. Data for the combined group of II and ID genotypes (II + ID) were compared with those for individuals with the DD genotype (recessive genetic model). Data for the combined group of ID and DD genotypes (ID + DD) were compared with those for individuals with the II genotype (dominant genetic model). ^bP=0.0325 versus II.

genotypes of *COL1A2* was in Hardy-Weinberg equilibrium and age did not differ among genotypes for men (Table VIII). Systolic BP was significantly higher in men with the SS genotype than in those in the combined group of SL and LL genotypes, whereas diastolic BP was significantly higher in men with the SS genotype than in those with the SL genotype or in those in the combined group of SL and LL genotypes. The differences in systolic and diastolic BP between individuals with the SS genotype and those in the combined group of SL and LL genotypes were 2.1 and 2.5%, respectively. The prevalence of hypertension did not differ among *COL1A2* genotypes for men. There were no differences in systolic or diastolic BP or in the prevalence of hypertension among *COL1A2* genotypes in women (data not shown).

Relation of the I/D (22,375delAC) polymorphism of CAV1 to BP. For men, the distribution of genotypes for the 22,375 I/D polymorphism of *CAVI* was in Hardy-Weinberg equilibrium and age did not differ among genotypes (Table IX). Systolic BP was significantly higher for men in the combined group of ID and DD genotypes than for those with the II genotype; the difference in systolic BP between these groups was 3.4%. The prevalence of hypertension did not differ among *CAVI* genotypes for men. For women, neither systolic or diastolic BP nor the prevalence of hypertension differed among *CAVI* genotypes (data not shown).

Relation of the -344C-T polymorphism of CYP11B2 to BP. The distribution of genotypes for the -344C-T polymorphism of *CYP11B2* was in Hardy-Weinberg equilibrium and age did not differ among genotypes for men (Table X). Systolic and diastolic BP were significantly higher in men with the CT genotype or with the TT genotype or in those in the combined group of CT and TT genotypes than in those with the CC genotype. The difference in systolic or diastolic BP between individuals in the combined group of CT and TT genotypes and those with the CC genotype was 4.9%. The prevalence of hypertension also differed significantly among genotypes (CC versus CT versus TT), being greater for men in the combined group of CT and TT genotypes than for those with the CC genotype. The odds ratio of the T allele compared with the C allele for predisposition to hypertension was 1.2. Although there were no differences in systolic or diastolic BP among *CYP11B2* genotypes in women (data not shown), the prevalence of hypertension differed among genotypes [CC (26.6%) versus CT (33.9%) versus TT (38.3%), P=0.0333], being greater for women in the combined group of CT and TT genotypes (36.0%) than for those with the CC genotype (26.6%, P=0.0272) as well as greater for women with the TT genotype (38.3%) than for those in the combined group of CC and CT genotypes (32.3%, P=0.0387). The odds ratio of the T allele compared with the C allele for predisposition to hypertension was 1.3.

Table X. Blood pressure (BP) for male subjects according to *CYP11B2* genotype.

Characteristics	CC	CT	TT	CC + CT	CT + TT
Number of subjects (n=876) ^a	109	418	349	527	767
Age (years)	59.5±1.0	59.1±0.5	59.2±0.5	59.2±0.4	59.1±0.4
Systolic BP (mmHg)	115.9±1.7	122.6±0.8 ^b	121.1±0.9 ^c	121.2±0.8	121.9±0.6 ^d
Diastolic BP (mmHg)	72.2±1.0	76.2±0.5 ^e	75.5±0.6 ^f	75.3±0.5	75.9±0.4 ^g
Number of all subjects (n=1125)	130	541	454	671	995
Hypertension (%)	23.9 ⁱ	35.3 ⁱ	36.6 ⁱ	33.1	35.9 ^h

^aSubjects not taking antihypertensive medication. Data for age and BP are means ± SE. Data for the combined group of CC and CT genotypes (CC + CT) were compared with those for individuals with the TT genotype (recessive genetic model). Data for the combined group of CT and TT genotypes (CT + TT) were compared with those for individuals with the CC genotype (dominant genetic model). ^bP=0.0010, ^cP=0.0171, ^dP=0.0007, ^eP=0.0014, ^fP=0.0115, ^gP=0.0007, ^hP=0.0053 versus CC. ⁱP=0.0188 (CC versus CT versus TT, 3 x 2 Chi-square test).

Table XI. Blood pressure (BP) for male subjects according to *ADRB1* genotype.

Characteristics	AA	AG	GG	AA + AG	AG + GG
Number of subjects (n=876) ^a	627	233	16	860	249
Age (years)	59.1±0.4	59.7±0.6	59.2±2.4	59.3±0.4	59.7±0.6
Systolic BP (mmHg)	120.6±0.7	122.6±1.1	125.9±4.3	121.1±0.6	122.8±1.1
Diastolic BP (mmHg)	75.0±0.4	76.4±0.7	79.5±2.7	75.4±0.4	76.6±0.7 ^b
Number of all subjects (n=1125)	804	301	20	1105	321
Hypertension (%)	33.7	35.9	45.0	34.3	36.5

^aSubjects not taking antihypertensive medication. Data for age and BP are means ± SE. Data for the combined group of AA and AG genotypes (AA + AG) were compared with those for individuals with the GG genotype (recessive genetic model). Data for the combined group of AG and GG genotypes (AG + GG) were compared with those for individuals with the AA genotype (dominant genetic model). ^bP=0.0430 versus AA.

Relation of the A-G (Ser49Gly) polymorphism of ADRB1 to BP. For men, the distribution of genotypes for the A-G polymorphism of *ADRB1* was in Hardy-Weinberg equilibrium and age did not differ among genotypes (Table XI). Diastolic BP was significantly higher for men in the combined group of AG and GG genotypes than for those with the AA genotype, the difference in diastolic BP between these groups being 2.1%. The prevalence of hypertension did not differ among *ADRB1* genotypes for men. Although systolic and diastolic BP did not differ among *ADRB1* genotypes for women (data not shown), the prevalence of hypertension differed significantly among genotypes [AA (32.6%) versus AG (41.4%) versus GG (29.6%), P=0.0270], being greater for women in the combined group of AG and GG genotypes (40.3%) than for those with the AA genotype (32.6%, P=0.0156). The odds ratio of the G allele compared with the T allele for predisposition to hypertension was 1.3.

Relation of the -1213T-C polymorphism of ESR2 to BP. In men, the distribution of genotypes for the -1213T-C polymorphism of *ESR2* was not in Hardy-Weinberg equilibrium and age did not differ among genotypes (Table XII). Systolic and diastolic BP were significantly higher in men with the TC genotype or in those in the combined group of TC and CC genotypes than in those with the TT genotype. The

differences in systolic and diastolic BP between individuals in the combined group of TC and CC genotypes and those with the TT genotype were 3.3 and 4.2%, respectively. The prevalence of hypertension did not differ among *ESR2* genotypes for men. For women, there was no difference in systolic or diastolic BP or in the prevalence of hypertension among *ESR2* genotypes (data not shown).

Discussion

The regulation of blood pressure involves the integration of a variety of biological systems that control the structure and tone of the vasculature as well as the volume and composition of body fluid. It also involves the adaptation of these systems to constantly changing physiological needs (16). We have now examined the relations of nine candidate gene polymorphisms to BP and the prevalence of hypertension in community-dwelling Japanese women and men. Our results show that the polymorphism of *FYN* was associated with systolic and diastolic BP in women as well as with systolic BP in men; the polymorphisms of *CNR2*, *QPCT*, *GPXI*, *COL1A2*, *CYP11B2*, and *ESR2* with systolic and diastolic BP in men; and those of *CAVI* and *ADRB1* with systolic and diastolic BP, respectively, in men. The polymorphisms of *QPCT* and *CYP11B2* were also associated with the prevalence

Table XII. Blood pressure (BP) for male subjects according to *ESR2* genotype.

Characteristics	<i>TT</i>	<i>TC</i>	<i>CC</i>	<i>TT + TC</i>	<i>TC + CC</i>
Number of subjects (n=879) ^a	773	97	9	870	106
Age (years)	59.4±0.3	57.4±1.0	61.7±3.3	59.2±0.4	57.7±0.9
Systolic BP (mmHg)	120.7±0.6	125.5±1.7 ^b	117.2±5.7	121.2±0.6	124.8±1.7 ^c
Diastolic BP (mmHg)	75.0±0.4	79.0±1.1 ^d	70.9±3.5	75.5±0.4	78.3±1.0 ^e
Number of all subjects (n=1128)	991	126	11	1117	137
Hypertension (%)	33.5	42.1	27.3	34.5	40.9

^aSubjects not taking antihypertensive medication. Data for age and BP are means ± SE. Data for the combined group of *TT* and *TC* genotypes (*TT + TC*) were compared with those for individuals with the *CC* genotype (recessive genetic model). Data for the combined group of *TC* and *CC* genotypes (*TC + CC*) were compared with those for individuals with the *TT* genotype (dominant genetic model). ^bP=0.0257, ^cP=0.0212, ^dP=0.0014, ^eP=0.0027 versus *TT*.

of hypertension in men. These observations thus suggest that polymorphisms of *QPCT* and *CYP11B2* are determinants of BP and the development of hypertension in Japanese men.

Given that selection bias can influence the results of genetic association studies, it is important that study populations be genetically and ethnically homogeneous. Our study subjects were recruited randomly from individuals residing in the city of Obu and town of Higashiura in central Japan, where the population is thought to share the same ethnic ancestry and to possess a homogeneous genetic background. We also showed that, with the exception of *ESR2*, the genotype distributions of the examined polymorphisms were in Hardy-Weinberg equilibrium in the study population. We thus appeared to avoid admixture and selection bias.

We detected associations of all nine polymorphisms with BP in men, whereas only the *FYN* polymorphism was associated with BP in women. The reason for this sex difference remains unclear. It might, however, be attributable, at least in part, to the difference in the plasma concentration of estrogen between men and women, given that estrogen exerts various favorable effects on vasomotor function, including stimulation of the production of nitric oxide and prostaglandin I₂ as well as inhibition of the release of endothelin-1 by vascular endothelial cells (17).

The formation of amino-terminal pyroglutamate from its glutaminy precursor is an important posttranslational or cotranslational event in the processing of numerous bioactive neuropeptides, hormones, and cytokines during their maturation in the secretory pathway. These regulatory peptides require the amino-terminal pyroglutamate to develop the correct conformation for binding to their receptors or to protect their amino termini from exopeptidase-mediated degradation (18,19). The glutaminy cyclases are acyl-transferases that catalyze this posttranslational modification (20,21). They are abundant in mammalian neuroendocrine tissues, such as the hypothalamus and pituitary gland (21,22), and are highly conserved from yeast to humans. In humans, the glutaminy-peptide cyclotransferase (glutaminy cyclase) gene (*QPCT*) is located at chromosomal position 2p22.2. Ezura *et al* (23) examined the relations of 13 polymorphisms in this region to bone mineral density (BMD) in adult women and detected associations between the genotypes of six polymorphisms and BMD for the radius. The 160C-T

(Arg54Trp) polymorphism of *QPCT* showed the most pronounced association, with the *T* allele being associated with low BMD. These results indicate that genetic variation in *QPCT* is an important determinant of BMD in adult women and may therefore contribute to susceptibility to osteoporosis. We have now shown that the 160C-T (Arg54Trp) polymorphism of *QPCT* was associated both with systolic and diastolic BP and with the prevalence of hypertension in Japanese men, with the *T* allele being associated with high BP. The effect of this polymorphism on gene expression or protein function has not been determined. This is the first demonstration of an association of this polymorphism of *QPCT* with BP or the prevalence of hypertension, but the underlying molecular mechanism of the association remains to be elucidated.

The cytochrome P450, subfamily Y XIB, polypeptide 2 (aldosterone synthase) gene (*CYP11B2*) encodes an enzyme that participates in the terminal steps of aldosterone synthesis in the zona glomerulosa cells of human adrenal glands, and its expression is regulated by angiotensin II and potassium (24). The candidacy of this gene in the present study is based on its pathogenic role in the syndrome of glucocorticoid-remediable aldosteronism (25). Several common polymorphisms of *CYP11B2* have been described (26-28). The -344C-T polymorphism, which is located in a putative binding site for a steroidogenic transcription factor, has been associated with hypertension (29-31) and with other hypertensive intermediate phenotypes such as plasma aldosterone level (32), urinary aldosterone excretion rate (30), and the aldosterone/renin ratio (27,28). Although some studies have not confirmed these relations (33,34), this locus may be important in the regulation of BP and the development of hypertension (35). We have now shown that the -344C-T polymorphism of *CYP11B2* was associated with both systolic and diastolic BP and the prevalence of hypertension in Japanese men, with the *T* allele being associated with high BP. Our results are thus consistent with previous observations (29-31).

Given the multiple comparisons of genotypes with BP or the prevalence of hypertension in the present study, it is not possible to completely exclude potential statistical errors such as false positives. It is also possible that one or more of the polymorphisms associated with BP or the prevalence of hypertension in our study are in linkage disequilibrium with

other polymorphisms of the same genes or of nearby genes that are actually responsible for the development of high BP. Furthermore, the relevance of the identified polymorphisms to gene transcription or to protein structure or function was not determined in the present study.

In conclusion, our results implicate *QPCT* and *CYP11B2* in the regulation of BP and the development of hypertension in Japanese men. Determination of genotypes for these polymorphisms may prove informative for assessment of the genetic risk for hypertension and may contribute to the personalized prevention of this condition. 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 essential hypertension, identification of additional hypertension susceptibility genes will allow more accurate assessment of the genetic component of this condition.

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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.

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,

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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	<i>PLOD1</i>	386G→A (Ala99Thr)	rs7551175
1p36.11	Cannabinoid receptor 2	<i>CNR2</i>	A→G	rs2501431
1p35.1	Gap junction protein, α -4	<i>GJA4</i>	1019C→T (Pro319Ser)	rs1764391
5q15	Adipocyte-derived leucine aminopeptidase	<i>ALAP</i>	1583G→A (Arg528Lys)	rs30187
15q21-23	Lipase, hepatic	<i>LIPC</i>	-514C→T	rs1800588
19p13.3-13.2	Intercellular adhesion molecule 1	<i>ICAM1</i>	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 μ l) contained 20 ng of DNA, 5 pmol of each primer, 0.2 mmol/l of each deoxy-

nucleoside 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 (intercalator-mediated fluorescence resonance energy transfer probe method). The polymorphic region of each gene was amplified by PCR (Table II) in a reaction mixture (25 μ l) 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 μ l) 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 \pm 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

Table II. Primers, probes, and other PCR conditions for genotyping.

Gene	Polymorphism	Sense primer with FITC	Sense primer with Texas red		
<i>GJA4</i>	1019C→T (Pro319Ser)	CCTCAGAATGGCCAAAAXTC	CTCAGAATGGCCAAAAXCC		
<i>ICAM1</i>	1462A→G (Lys469Glu)	AAGGGGAGGTCACCCGXGA	AGGGGAGGTCACCCGXAA		
		Antisense primer with biotin	Annealing (°C)	Cycles	Mg ²⁺ (mM)
<i>GJA4</i>		GCAGAGCTGCTGGGACGA	60	35	2.5
<i>ICAM1</i>		CTCACAGAGCACATTCACGGTCAC	60	35	4.5
Gene	Polymorphism	Sense primer	Antisense primer		
<i>PLOD1</i>	386G→A (Ala99Thr)	AAGCACGCAGACAAGGAGGATCTG	GAGGGCCTCATTTAGAAATATTCTTCTATCTTC		
<i>CNR2</i>	A→G	GGGCAGGTAGGAGACTAGTGCTGAGAG	CTCACCCGTGGAAGGGCACTG		
<i>ALAP</i>	1583G→A (Arg528Lys)	CCCTTCATGTAGTGCTCTTGCTTCATG	GCATCAGGAAGGGGTGGATGTG		
<i>LIPC</i>	-514C→T	GGGCATCTTTGCTTCTTCGTGTCAG	TTGGTGATGCTTGTGGTCAAAGTG		
		Probe	Annealing (°C)	Cycles	Mg ²⁺ (mM)
<i>PLOD1</i>		CATTCTCTTGGCAGACAGGTCAG	65	45	2.0
<i>CNR2</i>		CACATGATGCCAGGGTC	65	45	2.0
<i>ALAP</i>		CCCCTCTGCAGTGTCCAA	65	45	2.0
<i>LIPC</i>		TTCACCCCATGTCAAAA	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

^aBMD is adjusted for age, height, and body weight. Data are means ± SE. ^bP=0.0442, ^cP=0.0320, ^dP=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

^aBMD is adjusted for age, height, and body weight. Data are means ± SE. ^bP=0.0497, ^cP=0.0218, ^dP=0.0070, ^eP=0.0118, ^fP=0.0449, ^gP=0.0352, ^hP=0.0352, ⁱP=0.0412 versus GG; ^jP=0.0220, ^kP=0.0018, ^lP=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).

Table V. 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

^aBMD is adjusted for age, height, and body weight. Data are means ± SE. ^bP=0.0334, ^cP=0.0173, ^dP=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

^aBMD is adjusted for age, height, and body weight. Data are means ± SE. ^bP=0.0290, ^cP=0.0497, ^eP=0.0237, ^fP=0.0445 versus GG; ^dP=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

^aBMD is adjusted for age, height, and body weight. Data are means ± SE. ^bP=0.0370, ^cP=0.0181, ^dP=0.0011, ^eP=0.0029, ^fP=0.0004, ^gP=0.0085, ^hP=0.0042, ⁱP=0.0020, ^jP=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

^aBMD is adjusted for age, height, and body weight. Data are means ± SE. ^bP=0.0107, ^cP=0.0380, ^dP=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-

Table IX. 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

^aBMD is adjusted for age, height, and body weight. Data are means ± SE. ^bP=0.0310, ^cP=0.0098, ^dP=0.0324, ^eP=0.0353, ^fP=0.0317 versus TT; ^gP=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

^aBMD is adjusted for age, height, and body weight. Data are means ± SE. ^bP=0.0334, ^cP=0.0348 versus TT; ^dP=0.0173, ^eP=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

^aBMD is adjusted for age, height, and body weight. Data are means ± SE. ^bP=0.0148, ^cP=0.0046, ^dP=0.0142, ^eP=0.0439, ^fP=0.0489, ^gP=0.0101 versus GG; ^hP=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	AG + 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

^aBMD is adjusted for age, height, and body weight. Data are means ± SE. ^bP=0.0147, ^cP=0.0068, ^dP=0.0083, ^eP=0.0166, ^fP=0.0073, ^gP=0.0066, ^hP=0.0436 versus GG; ⁱP=0.0205, ^jP=0.0319, ^kP=0.0441, ^lP=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

between individuals with the *GG* genotype and the combined group of *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*, *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, 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 hydroxylysyl-pyridinoline 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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