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

[a]Subjects 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). [b]P=0.0313 versus AA; [c]P=0.0230, [d]P=0.0101, [e]P=0.0025, [f]P=0.0071, [g]P=0.0065, [h]P=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]

[a]Subjects 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). [b]P=0.0233, [c]P=0.0434, [d]P=0.0010 versus CC. [e]P=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 the160C→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 GPX1 to BP. Among men, the distribution of genotypes for the C→T (Pro198Leu) polymorphism of *GPX1* 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 *GPX1* genotypes for men. Among women, no difference in systolic or diastolic BP or in the prevalence of hypertension was detected among *GPX1* 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

[a]Subjects 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). [b]P=0.0188, [c]P=0.0072, [d]P=0.0078, [e]P=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

[a]Subjects 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). [b]P=0.0217, [c]P=0.0138, [d]P=0.0132, [e]P=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

[a]Subjects 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). [b]P=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 COL1A2 to BP. Given that the mean and median numbers of GT repeats for *COL1A2* in the study subjects were 14.2 and 12, respectively, we designated $(GT)_{n\leq12}$ and $(GT)_{n\geq13}$ 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

[a]Subjects not taking antihypertensive medication. Data for age and BP are means ± SE. *S*, short repeat allele [$(GT)_n \leq 12$]; *L*, long repeat allele [$(GT)_n \geq 13$]. 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). [b]P=0.0460, [c]P=0.0341, [d]P=0.0178 versus *SS*.

Table IX. Blood pressure (BP) for male subjects according to *CAV1* 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

[a]Subjects 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). [b]P=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 *CAV1* 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 *CAV1* genotypes for men. For women, neither systolic or diastolic BP nor the prevalence of hypertension differed among *CAV1* 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]

[a]Subjects 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). [b]P=0.0010, [c]P=0.0171, [d]P=0.0007, [e]P=0.0014, [f]P=0.0115, [g]P=0.0007, [h]P=0.0053 versus *CC*. [i]P=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

[a]Subjects 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). [b]P=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*, *GPX1*, *COL1A2*, *CYP11B2*, and *ESR2* with systolic and diastolic BP in men; and those of *CAV1* 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	*TT*	*TC*	*CC*	*TT + TC*	*TC + CC*
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

[a]Subjects 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). [b]P=0.0257, [c]P=0.0212, [d]P=0.0014, [e]P=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 I_2 as well as inhibition of the release of endothelin-1 by vascular endothelial cells (17).

The formation of amino-terminal pyroglutamate from its glutaminyl 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 glutaminyl cyclases are acyltransferases 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 glutaminyl-peptide cyclotransferase (glutaminyl 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.

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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

Table II. 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).

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

[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-

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

[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*	*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]

[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

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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Effects of the interaction between lean tissue mass and estrogen receptor α gene polymorphism on bone mineral density in middle-aged and elderly Japanese

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Abstract

Because both genetic and environmental factors influence bone mass, it is important to examine the effect of gene-environment interactions on bone mineral density (BMD) for the prevention of osteoporosis at an individual level. Estrogen receptor α (ERα) plays an important role in increasing BMD via mechanical strain and muscle mass is a reflection of the forces the muscle applies to the bone. The aim of this study is to investigate the effect of the interaction between lean tissue mass (LTM) and the ERα polymorphisms T→C (*PvuII*) [dbSNP: rs2234693] and A→G (*XbaI*) [dbSNP: rs9340799] on BMD in middle-aged and elderly individuals. Subjects were 2209 community-dwelling Japanese men and women, ages 40 to 79 years. ERα polymorphisms in the first intron, T→C and A→G were identified and lumbar spine and femoral neck BMD and LTM were measured by dual-energy X-ray absorptiometry. Both T→C and A→G polymorphisms were divided into two genotype groups (TT vs. TC/CC; AA vs. AG/GG). In postmenopausal women, the effect of LTM on femoral neck BMD was significantly larger for those with the TC/CC genotype than for those with the TT genotype for the T→C polymorphism, and larger for those with the AG/GG genotype than for those with the AA genotype for the A→G polymorphism. This gene–LTM interaction was observed at the femoral neck, but not at the lumbar spine. For men and premenopausal women, no gene–LTM interaction was found. In conclusion, there was an interaction between LTM and the ERα T→C and A→G polymorphisms with respect to their effect on femoral neck BMD in postmenopausal women and those with the TC/CC and AG/GG genotypes had larger effects of LTM than those with TT and AA genotypes.
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Keywords: Single nucleotide polymorphism; Estrogen receptor alpha; BMD; Lean tissue mass; Postmenopausal women

Introduction

It is generally accepted that dynamic loading acts as an osteogenic stimulus [1] and that the forces applied to bone are primarily the result of muscular contraction [2]. Therefore, muscular weakness is an important factor contributing to osteoporosis [3]. The importance of skeletal muscle in preserving bone [4] and the relation between low skeletal mass and poor structural parameters of bone in elderly men [5] have been reported. A previous study suggested that physical exercise maintains bone mineral density (BMD) in postmenopausal women [6]. Vainionpaa et al. showed that the intensity of exercise was significantly correlated with BMD changes [7] and Kerr et al. reported that postmenopausal bone mass can be significantly increased by strength training, but not by endurance training [8].

Animal studies have suggested that mechanical strain stimulates osteoblast proliferation through estrogen receptor α (ERα) [9], and osteoblast-like cells from ERα knockout mice have deficient responses to mechanical strain [10]. Thus, it is thought that ERα plays an important role in increasing BMD via mechanical strain [11,12]. Although the association between ERα genotype and the risk of osteoporosis in humans remains controversial [13], many studies have suggested a

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relation between ERα polymorphism and BMD [14–16]. A study previously carried out in our laboratory also showed that the ERα gene was a susceptibility locus for reduced bone mass, especially at the femoral neck, in elderly Japanese women [17].

Because the effects of environment on individuals might differ in accordance with individuals' different genetic makeups, it is important to examine the effects of the gene-environment interaction on BMD, particularly for the prevention of osteoporosis at an individual level. Some studies have investigated the effect of ERα polymorphism on the relationship between exercise and BMD. These studies have shown an effect of the ERα gene (*PvuII*)–exercise interaction on BMD in middle-aged men [18] and prepubertal and early pubertal girls [19].

Because magnetic resonance imaging (MRI)-measured muscle area correlates with muscle strength [20], and the differences between MRI-measured and dual-energy X-ray absorptiometry (DXA)-predicted skeletal muscle mass are small [21], DXA-predicted total body lean mass can be legitimately used as an index of skeletal load. As mentioned above, a few studies have investigated the effects of the ERα gene–exercise interaction on BMD. However, the effects of the ERα gene–lean tissue mass (LTM) interaction were unknown. Furthermore, these previous studies involved single-sex populations within a limited age range. This study investigated for the first time the effects of the interaction between LTM and the typical ERα polymorphisms T→C (*PvuII*) and A→G (*XbaI*) on BMD in both men and women in a large population.

Materials and methods

Subjects

Study subjects were 1119 men and 1090 women, ages 40–79 years, who participated in the first wave (from April 1998 to March 2000) of the National Institute for Longevity Sciences-Longitudinal Study of Aging (NILS-LSA), which is a population-based prospective cohort study of aging and age-related diseases. Participants in the NILS-LSA were randomly selected age and sex stratified individuals selected from the pool of independent residents in the NILS neighborhood, Obu city and Higashiura town, Aichi Prefecture, central Japan. Details of the NILS-LSA have been given elsewhere [22]. The study protocol was approved by the Committee of Ethics of Human Research of the National Center for Geriatrics and Gerontology. Written informed consent was obtained from all subjects.

Anthropometric variables

Body weight was measured to the nearest 0.01 kg using digital scales, height was measured to the nearest 0.1 cm using a stadiometer, and body mass index (BMI) was calculated as weight (kg) divided by height squared (m^2).

Menstrual status

Menopause was confirmed as the absence of menses by a questionnaire.

Dual-energy X-ray absorptiometry

Whole-body fat mass, LTM, bone mineral content (BMC), and BMD of the femoral neck and lumbar spine (L2–4) were assessed by DXA (QDR-4500; Hologic, Madison, OH, USA). Lean tissue mass is equal to the fat-free mass minus BMC, and is assumed to be an index of the amount of muscle mass.

ERα genotype analysis

DNA was extracted from peripheral blood lymphocytes by using a standard procedure. ERα genotypes were determined in accordance with a study by Yamada et al. [17] The ERα genotypes were analyzed by using an automated fluorescent allele-specific DNA primer assay system (Toyobo Gene Analysis, Tsuruga, Japan). To determine the T→C (*PvuII*) genotype, the polymorphic region of the gene was amplified by polymerase chain reaction (PCR) using allele-specific sense primers labeled at the 5′ end either with fluorescein isothiocyanate (5′-AGTTCCAAATGTCCCAGXTG-3′) or with Texas red (5′-AGTTCCAAATGTCCCAGXCG-3′) and an antisense primer labeled at the 5′ end with biotin (5′-TCTGGGAAACAGAGACAAAGC-3′). The reaction mixture (25 μl) contained 20 ng DNA, 5 pmol of each primer, 0.2 mmol/l of each deoxynucleoside triphosphate, 2.5 mmol/l $MgCl_2$, and 1U DNA polymerase (rTaq; Toyobo, Osaka, Japan) in rTaq buffer. The amplification protocol consisted of initial denaturation at 95 °C for 5 min; 35 cycles of denaturation at 95 °C for 30 s, annealing at 62.5 °C for 30 s, and extension at 72 °C for 30 s; and a final extension at 72 °C for 2 min. For determination of the A→G (*XbaI*) genotype, the polymorphic region of the gene was amplified by PCR using a sense primer labeled at the 5′ end with biotin (5′-CTGTTTCCCA-GAGACCCTGAG-3′) and allele-specific antisense primers labeled at the 5′ ends either with fluorescein isothiocyanate (5′-CCAATGCTCAT-CCCAACTXTA-3′) or with Texas red (5′-CCAATGCTCATCCCAACTXCA-3′). The reaction mixture (with the exception of the primers) and the amplification protocol (with the exception that the annealing temperature was 65 °C) were identical to those used for genotyping of the T→C polymorphism.

Amplified DNA was incubated in a solution containing streptavidin-conjugated magnetic beads in the wells of a 96-well plate at room temperature. The plate was placed on a magnetic stand and the supernatant was discarded. After two washings, 0.01 M NaOH was added to the wells and mixed well. The plate was placed on a magnetic stand again and the supernatants were transferred to the wells of a new 96-well plate. The fluorescence was measured by using a microplate reader (Fluoroscan Ascent; Dainippon Pharmaceutical, Osaka, Japan) at excitation and emission wavelengths of 485 nm and 538 nm, respectively, for fluorescein isothiocyanate, and 584 nm and 612 nm, respectively, for Texas red.

Haplotype analysis

The haplotype distribution was calculated by using Haplotyper, a software program for haplotype inference, with the Bayesian algorithm [23,24].

Statistical analysis

Values are expressed as the mean±standard error (SE). The chi-squared test was used to identify significant departures from Hardy-Weinberg equilibrium. Both T→C and A→G polymorphisms were divided into two genotype groups (TT vs. TC/CC; AA vs. AG/GG). The differences between genotype groups were analyzed using one-way analysis of variance and the Tukey–Kramer post hoc test. A general linear model was employed to evaluate the effect of the LTM–genotype interaction on BMD (adjusted for age and BMI). When the effect of the interaction on BMD was significant for both T→C and A→G polymorphisms, further analysis (in accordance with haplotype groups) was

Table 1
Distribution of T→C and A→G genotypes of the ERα gene

	AA		AG		GG		Total	
	n	%	*n*	%	*n*	%	*n*	%
TT	787	35.6	1	0.1	0	0.0	788	35.7
TC	584	26.4	465	21.1	5	0.2	1054	47.7
CC	120	5.4	174	7.9	73	3.3	367	16.6
Total	1491	67.5	640	29.0	78	3.5	2209	100.0

Table 2
Physical characteristics of subjects with reference to the T→C and A→G genotypes of the ERα gene

	Men (n=1119)		Premenopausal women (n=278)		Postmenopausal women (n=812)	
	TT (n=398)	TC/CC (n=721)	TT (n=98)	TC/CC (n=180)	TT (n=292)	TC/CC (n=520)
Age (years)	58.9±0.6	59.3±0.4	46.2±0.5	46.2±0.3	62.8±0.5	64.6±0.4*
Weight (kg)	62.9±0.5	62.2±0.3	53.9±0.8	54.7±0.6	52.5±0.5	51.7±0.4
BMI (kg/m2)	23.2±0.1	22.9±0.1	22.5±0.3	22.9±0.2	23.1±0.2	23.0±0.2
LTM (kg)	47.2±0.3	46.6±0.2	36.3±0.4	36.5±0.3	33.9±0.2	33.7±0.2
L2–4 BMD (kg/cm^2)	0.99±0.01	0.98±0.01	1.03±0.01	1.02±0.01	0.82±0.01	0.80±0.01
Femoral neck BMD (g/cm^2)	0.76±0.01	0.75±0.004	0.78±0.01	0.77±0.01	0.66±0.01	0.64±0.004*
	AA (n=769)	AG/GG (n=350)	AA (n=192)	AG/GG (n=86)	AA (n=530)	AG/GG (n=282)
Age (years)	59.2±0.4	59.1±0.5	46.3±0.3	46.0±0.5	63.7±0.4	64.2±0.5
Weight (kg)	62.7±0.3	61.9±0.5	53.5±0.5	56.4±1.0	51.9±0.3	52.2±0.5
BMI (kg/m^2)	23.1±0.1	22.8±0.1	22.3±0.2	23.7±0.4**	22.9±0.1	23.3±0.2
LTM (kg)	47.0±0.2	46.5±0.3	36.1±0.3	37.0±0.5	33.8±0.2	33.7±0.2
L2–4 BMD (kg/cm^2)	0.99±0.01	0.97±0.01	1.03±0.01	1.02±0.01	0.81±0.01	0.81±0.01
Femoral neck BMD (g/cm^2)	0.75±0.004	0.75±0.01	0.77±0.01	0.78±0.01	0.65±0.004	0.64±0.01

Data are mean±SE. *$p<0.05$ vs. TT genotype, **$p<0.01$ vs. AA genotype.

carried out. Values of $p<0.05$ were considered to indicate statistical significance. Data were analyzed with the Statistical Analysis System (SAS) release 8.2 (SAS Institute Inc., Cary, NC, USA).

Results

Distribution of ERα genotypes

The distribution of genotype combinations was examined (Table 1). The distributions of ERα T→C and A→G genotypes were both in Hardy–Weinberg equilibrium. There were no subjects with the TT and GG genotypic combination and few with the TT/AG or TC/GG genotypic combination.

Physical characteristics

Physical characteristics of the subjects were compared with reference to the ERα T→C and A→G genotype groups (Table 2). For men and premenopausal women, age, weight, BMI, LTM, L2–4 BMD, and femoral neck BMD did not differ between subjects with the TT and TC/CC genotypes. In contrast, in postmenopausal women, age was significantly higher and femoral neck BMD was significantly lower in individuals with the TC/CC genotype than in those with the TT genotype. After adjusting for age, statistical significance was not achieved for the difference in femoral neck BMD in postmenopausal women (data not shown). In men and postmenopausal women, there were no differences in age and physical characteristics between subjects with the AA and AG/GG genotypes. In premenopausal women, age, weight, LTM, and BMD did not differ between subjects with the AA and AG/GG genotypes, whereas BMI was significantly greater in those with the AG/GG genotype than in those with the AA genotype. After adjusting for BMI, the relationship of L2–4 and femoral neck BMD between AA and AG/GG genotypes still did not show a significant difference in premenopausal women (data not shown).

ERα genotype and association between LTM and BMD

To investigate whether an interaction between ERα genotype and LTM had an effect on L2–4 and femoral neck BMDs, general linear models for BMD were analyzed using LTM, ERα

Table 3
General linear model for bone mineral density (BMD) with interaction between the ERα genotype and LTM

Dependent variables	Independent variables	Men		Premenopausal women		Postmenopausal women	
		F	p value	F	p value	F	p value
L2–4 BMD	LTM	45.65	<0.0001	24.73	<0.0001	25.53	<0.0001
	T→C genotype	0.91	ns	1.36	ns	2.41	ns
	LTM×(T→C genotype)	0.83	ns	1.29	ns	2.55	ns
Femoral neck BMD	LTM	63.90	<0.0001	15.07	<0.0001	25.35	<0.0001
	T→C genotype	0.03	ns	0.13	ns	8.15	0.004
	LTM×(T→C genotype)	0.03	ns	0.06	ns	7.48	0.007
L2–4 BMD	LTM	45.27	<0.0001	24.36	<0.0001	25.41	<0.0001
	A→G genotype	0.10	ns	0.16	ns	2.20	ns
	LTM×(A→G genotype)	0.05	ns	0.26	ns	2.14	ns
Femoral neck BMD	LTM	64.07	<0.0001	14.95	<0.0001	24.95	<0.0001
	A→G genotype	0.38	ns	0.07	ns	8.15	0.004
	LTM×(A→G genotype)	0.45	ns	0.05	ns	8.03	0.005

ns=not significant. Adjusted for age and BMI.