

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 *GPXI*, 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*, *GPXI*, *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.

Materials and methods

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

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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	Glutamyl-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>CAVI</i>	I/D (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*, *CAVI*, 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>		CCTTCCTCATGCCCTAAT	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)	AAAGGTGATGGATCATTCCCATTATACAC	TGGGCAATGGTCATCCATGACTG			
<i>ESR2</i>	-1213T-C	GAACAGGAGCCAGGGGCACAG	CCTGAAGACAAGTACCTTGCAGCTGAG			
		Probe	Annealing (°C)	Cycles	Mg ²⁺ (mM)	Taq/KOD
<i>CNR2</i>		CACATGATGCCCAGGGTC	65.0	45	2.0	Taq
<i>CAVI</i>		CAAAATGTGTGTCCATTTTCAGG	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>COL1A2</i>	(GT) _n	CAGCACGGTGTCTACCACTGC	ATTACTCCTTAGTATCCACAGTATGTATAC			
		Annealing (°C)	Cycles	Mg ²⁺ (mM)	Taq/KOD	
<i>COL1A2</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 *COL1A2* 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. BP 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 ^c	121.6±0.7
Diastolic BP (mmHg)	74.8±0.6 ^f	74.9±0.5 ^e	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 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 *GPXI* 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 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≤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 11D (22,375delAC) polymorphism of CAV1 to BP. For men, the distribution of genotypes for the 22,375 *ID* 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*, *GPX1*, *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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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 (ER α) 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 ER α polymorphisms T \rightarrow C (*PvuII*) [dbSNP: rs2234693] and A \rightarrow G (*AbaI*) [dbSNP: rs9340799] on BMD in middle-aged and elderly individuals. Subjects were 2209 community-dwelling Japanese men and women, ages 40 to 79 years. ER α polymorphisms in the first intron, T \rightarrow C and A \rightarrow G were identified and lumbar spine and femoral neck BMD and LTM were measured by dual-energy X-ray absorptiometry. Both T \rightarrow C and A \rightarrow 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 \rightarrow C polymorphism, and larger for those with the AG/GG genotype than for those with the AA genotype for the A \rightarrow 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 ER α T \rightarrow C and A \rightarrow 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 (ER α) [9], and osteoblast-like cells from ER α knockout mice have deficient responses to mechanical strain [10]. Thus, it is thought that ER α plays an important role in increasing BMD via mechanical strain [11,12]. Although the association between ER α genotype and the risk of osteoporosis in humans remains controversial [13], many studies have suggested a

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relation between ER α polymorphism and BMD [14–16]. A study previously carried out in our laboratory also showed that the ER α 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 make-ups, 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 ER α polymorphism on the relationship between exercise and BMD. These studies have shown an effect of the ER α 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 ER α gene-exercise interaction on BMD. However, the effects of the ER α 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 ER α polymorphisms T \rightarrow C (*PvuII*) and A \rightarrow 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 (m²).

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.

ER α genotype analysis

DNA was extracted from peripheral blood lymphocytes by using a standard procedure. ER α genotypes were determined in accordance with a study by Yamada et al. [17]. The ER α genotypes were analyzed by using an automated fluorescent allele-specific DNA primer assay system (Toyobo Gene Analysis, Tsuruga, Japan). To determine the T \rightarrow 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'-TCTGGGAAACAGACAAAAGC-3'). The reaction mixture (25 μ l) contained 20 ng DNA, 5 pmol of each primer, 0.2 mmol/l of each deoxynucleoside triphosphate, 2.5 mmol/l MgCl₂, 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 \rightarrow 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'-CTGTTCCCA-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 \rightarrow 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 \pm standard error (SE). The chi-squared test was used to identify significant departures from Hardy-Weinberg equilibrium. Both T \rightarrow C and A \rightarrow 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 \rightarrow C and A \rightarrow G polymorphisms, further analysis (in accordance with haplotype groups) was

Table 1
Distribution of T \rightarrow C and A \rightarrow G genotypes of the ER α 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/m ²)	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	<i>p</i> value	F	<i>p</i> value	F	<i>p</i> 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.

genotype, and the interaction between ER α genotype and LTM as independent variables, adjusting for age and BMI (Table 3). Lean tissue mass was significantly associated with L2–4 and femoral neck BMDs in both sexes and irrespective of menstrual status. In postmenopausal women, genotype and the interaction between genotype and LTM were significantly associated with femoral neck BMD for both the T \rightarrow C and A \rightarrow G genotypes, but not with L2–4 BMD. In men and premenopausal women, the effects of genotype and the interaction between genotype and LTM on BMD were not significant.

To clarify the influence of LTM on femoral neck BMD for T \rightarrow C and A \rightarrow G ER α genotypes in postmenopausal women, a general linear model for BMD was analyzed by each genotype, using LTM as an independent variable, adjusting for age and BMI. Fig. 1 shows the regression lines between femoral neck

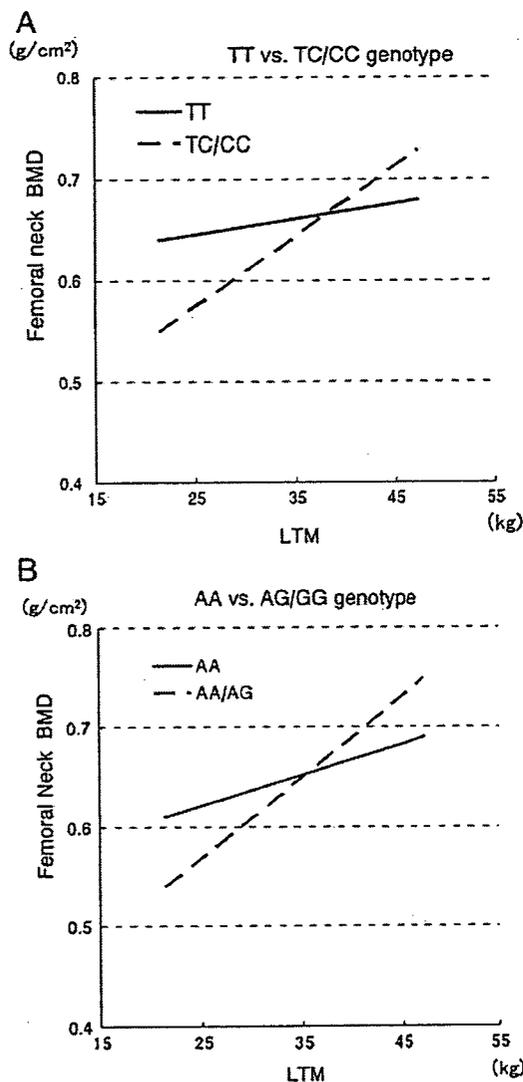


Fig. 1. Relationship between femoral neck BMD and LTM with reference to ER α T \rightarrow C and A \rightarrow G genotypes in postmenopausal women, adjusted for age and BMI. (A) T \rightarrow C genotype. Solid line, TT genotype; Dotted line, TC/CC genotype. (B) A \rightarrow G genotype. Solid line, AA genotype; Dotted line, AG/GG genotype.

Table 4

Physical characteristics of postmenopausal women with reference to ER α T \rightarrow C and A \rightarrow G haplotype

	Haplotype		
	TA	CA	CG
N	965	349	307
Age (years)	63.4 \pm 0.3	64.8 \pm 0.5*	64.3 \pm 0.5
Weight (kg)	52.2 \pm 0.3	51.6 \pm 0.4	51.8 \pm 0.5
BMI (kg/m ²)	23.0 \pm 0.1	22.9 \pm 0.2	23.2 \pm 0.2
LTM (kg)	33.8 \pm 0.1	33.7 \pm 0.2	33.6 \pm 0.2
BMD L2–4 (g/cm ²)	0.81 \pm 0.004	0.80 \pm 0.01	0.80 \pm 0.01
BMD femoral neck (g/cm ²)	0.65 \pm 0.003	0.64 \pm 0.01	0.64 \pm 0.01

Data are mean \pm SE. * p <0.05 vs. TA.

BMD and LTM for the ER α T \rightarrow C and A \rightarrow G genotype groups in postmenopausal women. For the T \rightarrow C genotype (Fig. 1A), the slope was significantly larger (p <0.01) for TC/CC (slope=0.0071, p <0.0001) than for TT individuals (slope=0.0015, not significant). For the A \rightarrow G genotype (Fig. 1B), the slope was significantly larger (p <0.01) for AG/GG (slope=0.0081, p <0.0001) than for AA individuals (slope=0.0033, p =0.012).

ER α haplotype and association between LTM and BMD in postmenopausal women

Because there were significant genotype-LTM interactions on femoral neck BMD for both T \rightarrow C and A \rightarrow G polymorphisms in postmenopausal women, further analysis was carried out to evaluate the effect of the haplotype-LTM interaction. The distribution of haplotypes is shown in Table 4. The possible haplotype combinations for the ER α T \rightarrow C and A \rightarrow G polymorphisms were TA, CA, TG, and CG, but very few subjects had the TG haplotype. For postmenopausal women, the number of TA haplotype was 965; CA was 349; and CG was 307. Physical characteristics and BMD were compared with reference to these three haplotypes (Table 4). Age was significantly higher for those with the CA haplotype than for those with the TA haplotype. Weight, BMI, LTM, and L2–4 and femoral neck BMDs did not differ among haplotypes.

To clarify the influence of the interaction between ER α haplotype and LTM on femoral neck BMD in postmenopausal women, general linear models for BMD were analyzed using LTM, ER α haplotype, and the interaction between ER α haplotype and LTM as independent variables, adjusting for age and BMI (Table 5). Lean tissue mass, haplotype, and the interaction between haplotype and LTM were significantly

Table 5

General linear model for femoral neck BMD with interactions between ER α haplotype and LTM in postmenopausal women

Dependent variables	Independent variables	Postmenopausal women	
		F	p
Femoral neck BMD	LTM	49.80	<0.0001
	haplotype	6.63	0.001
	LTM \times haplotype	6.23	0.002

Adjusted for age and BMI.

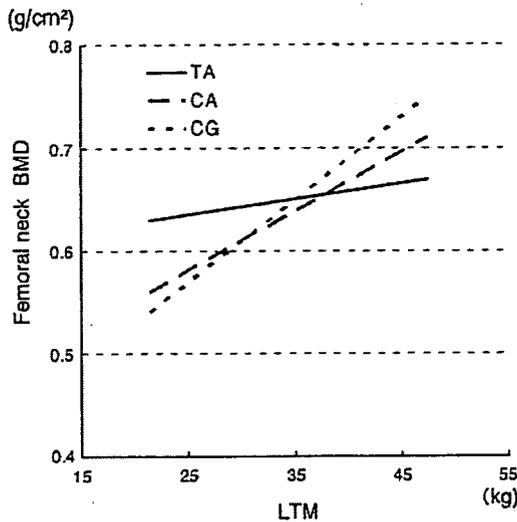


Fig. 2. Relationship between femoral neck BMD and LTM with reference to ER α haplotype in postmenopausal women, adjusted for age and BMI. Solid line, TA haplotype; Dotted line, CA haplotype; Fine dotted line, CG haplotype.

associated with femoral neck BMD. To evaluate the extent of the influence of LTM on BMD with respect to different haplotypes, general linear models were analyzed by each haplotype, using LTM as an independent variable, adjusting for age and BMI. Fig. 2 presents the relationship between femoral neck BMD and LTM with respect to the ER α haplotypes in postmenopausal women. The slope was significantly larger ($p < 0.01$) for subjects with the CG (slope = 0.0081, $p < 0.0001$) and CA (slope = 0.0063, $p < 0.0001$) haplotypes than for those with the TA haplotype (slope = 0.0035, $p = 0.0003$), but there was no difference between the CG and CA haplotypes.

Discussion

We found that ER α polymorphisms influence the relationship between LTM and femoral neck BMD in postmenopausal women and that the effect of LTM on BMD was significantly larger in individuals with the TC/CC genotype of the T \rightarrow C polymorphism than in those with the TT genotype, and larger in those with the AG/GG genotype of the A \rightarrow G polymorphism than in those with the AA genotype. Haplotype analysis revealed that the effect was significantly larger for those with the CG and CA haplotypes than for those with the TA haplotype. This is the first study to investigate the effect of ER α gene–LTM interaction on BMD and to reveal the significant interaction in postmenopausal women.

In this study, a significant gene–LTM interaction in postmenopausal women was found at the femoral neck, but not at the lumbar spine. It has been reported that hip joint compression forces reach 2.5 to 3 times body weight during walking [25,26]. The significant results found only at the femoral neck could be explained by the fact that high loading occurs at this site even in ordinary daily life.

We also analyzed data for different combinations of genotypes (TC/TT vs. CC; AG/AA vs. GG) (data not shown).

In premenopausal women, no significant gene–LTM interaction was found. In postmenopausal women, significant interaction was found at femoral neck between TT/TC and CC genotype groups; however, no significant genotype–LTM interaction was found between GG and AA/AG genotype groups. This might be due to the small number in the GG genotype group ($n = 28$). In men, when divided into TT/TC and CC genotypes, significant interaction was found at L2–4 and the femoral neck (L2–4, $p = 0.04$; femoral neck, $p = 0.02$) and the effect of LTM on BMD was larger for those with CC genotype than for those with TC/TT genotype. However, these significant interactions in men were weak in spite of the large number and the coefficients of determination (R^2) in the analysis model were low in men compared with postmenopausal women (men at the femoral neck, 0.28; men at L2–4, 0.18; postmenopausal women at the femoral neck, 0.38). Therefore, there might be other related factors in men and we considered that these results in men are insufficient to draw a clear conclusion about the effect of ER α gene–LTM interaction between TT/TC and CC genotype groups. We will examine this problem by adding other factors and analyzing the data longitudinally.

There have been some human studies investigating the effects of ER α gene polymorphism on exercise-induced effects on BMD. In a 4-year exercise intervention study, Remes et al. [18] reported that middle-aged Finnish men with the Pp (TC) or PP (CC) ER α genotype had increased lumbar spine BMD values. In the study of Remes et al., the subjects were middle-aged men, and the exercise intervention group spent 45–60 min on prescribed aerobic exercise five times a week for 4 years. Because we did not intervene as in the research of Remes et al., there might not have been a significant interaction between TT and TC/CC genotype groups in men.

Suuriniemi et al. [19] found that prepubertal and early pubertal Finnish girls with the Pp (TC) ER α genotype and high levels of physical activity had significantly higher bone mass and BMD at loaded bone sites. The subjects were 10- to 13-year-old prepubertal and early pubertal girls, whose estrogen concentrations were low, like those of postmenopausal women. A previous report indicated that estrogen can affect bone strength and mass by lowering the remodeling threshold, and that loss of estrogen would raise the threshold and help cause postmenopausal bone loss [27]. ER α expression in osteoblasts and osteocytes depends on estrogen concentration [28]. The increase in the potential of mechanical loading to stimulate bone gain in the peripubertal period is associated with marked increases in serum estrogen [11]. Thus, in the peripubertal period, when estrogen concentrations are high, the response to mechanical loading might be greater than in the prepubertal and postmenopausal periods, when estrogen concentrations are low. However, in the present study, significant interactions were found for postmenopausal women and in the study of Suuriniemi et al. [19] significant interactions were found for prepubertal and early pubertal girls. Accordingly, the effect of the gene–LTM interaction on BMD, that is, differences between individuals with different single nucleotide polymorphisms, might more readily appear in groups with low estrogen concentrations than in those with high concentrations.

In the study of Suuriniemi et al. [19], the interaction was found only for individuals with the Pp (TC) genotype (heterozygotes). This was not in agreement with our results. In this previous study, girls with low levels of physical activity bearing the Pp genotype had lower values for bone parameters compared with other groups. Because there are differences in the subject characteristics, age, sex, lifestyle, and study design, it is difficult to simply compare the results of the present study with those of the study by Suuriniemi et al. Further investigations are necessary to clarify these differences.

The mechanisms by which the ER α T \rightarrow C and A \rightarrow G polymorphisms might affect the femoral neck BMD are not clear, because the affected regions lie in an intronic and non-functional area of the gene. However, single nucleotide polymorphisms are usually linked to each other, so the two polymorphisms in intron 1 may be in linkage disequilibrium with causal polymorphisms elsewhere in the ER α gene or in genes nearby. In this regard, it is known that the T \rightarrow C and A \rightarrow G polymorphisms are in linkage disequilibrium with an upstream TA repeat polymorphism in the promoter region of the ER α gene [29]. An association between the TA repeat polymorphism and BMD has been shown in postmenopausal Japanese [30] and Italian women [29]. The number of TA repeats could be important in ER α gene transcription [31].

There are conflicting results regarding the association between ER α genotype and BMD in previous studies. Gene–LTM interactions might be one of the reasons for these differing results. That is, differences in the amount of muscle mass might change the association between ER genotype and BMD. For example, as shown in Fig. 1A, it is considered that in the group with low LTM, postmenopausal women with the TT genotype for T \rightarrow C polymorphism have higher femoral neck BMD than those with the CC genotype. Conversely, in the group with high LTM, postmenopausal women with the CC genotype have higher BMD than those with the TT genotype.

In the present study, we evaluated the relationship between LTM and BMD with reference to ER α genotype, but we did not evaluate cause and effect directly. A previous exercise intervention study [18] showed an interaction between ER α genotype and exercise (i.e. the effect of mechanical loading) on BMD in men. Because LTM correlates with muscle strength [20] and it can be used as an index of skeletal load, the result of this previous exercise intervention study supports our present results regarding the gene–LTM interaction.

The strengths of the present study are the large sample size, the inclusion of both sexes, and the wide range of ages. Previous research has evaluated only one sex and a limited age range [18,19]. So far, the ER α gene–environment interaction concerning mechanical loading has not been investigated in postmenopausal women, who are particularly susceptible to osteoporosis. In the present study, both T \rightarrow C and A \rightarrow G ER α polymorphisms were examined and haplotype analysis was carried out.

As already described, previous studies have investigated the effects of ER α gene polymorphism on exercise-induced effects on BMD, but there has been no study that has evaluated the effect of the ER α gene–LTM interaction on BMD. The well-

known phenomenon of reduction in muscle mass with aging is known as sarcopenia. Recent studies have reported a high prevalence of sarcopenia in postmenopausal woman with osteoporosis [32]. It would be very useful to identify individuals in this group who would experience a marked effect from increasing muscle mass, and the results of this study might assist in developing this process.

ER α plays an important role in the increase of BMD via mechanical strain [9–11]. A recent study has suggested that the effect of chronic immobility might be more marked on bone formation than bone resorption [33]. On the basis of our results, we can speculate that the influence of the mechanical loading increase on BMD via ER is different according to the ER α T \rightarrow C and A \rightarrow G polymorphisms in postmenopausal women.

In conclusion, there was an interaction between LTM and the T \rightarrow C and A \rightarrow G ER α 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.

Acknowledgments

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Brief Genetic Analysis

No Association between rs7566605 Variant and Being Overweight in Japanese

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Abstract

KUZUYA, MASAFUMI, FUJIKO ANDO, AKIHISA IGUCHI, AND HIROSHI SHIMOKATA. No association between rs7566605 variant and being overweight in Japanese. *Obesity*. 2007;15:2531–2534.

It has recently been demonstrated that a common single-nucleotide polymorphism (rs7566605) upstream of the transcription start site for the insulin-induced gene 2 is associated with obesity in several European/European origin or African-American cohorts. We tested whether this variant is also linked to overweight among Asian populations. Our sample included 2233 randomly selected, community-dwelling, middle-age and older Japanese people (men, 1128; women, 1105; age, 40 to 79 years; C allele frequency, 0.32). We observed that there were no differences in BMI levels [men, 22.9 ± 0.3 (mean \pm standard error) vs. 22.9 ± 0.1 , $p = 0.820$; women, 22.8 ± 0.3 vs. 22.9 ± 0.1 , $p = 0.792$], waist circumferences and hip circumferences, waist-to-hip ratio, and fat mass between rs7566605 GG/GC and CC genotypes in both genders. In addition, logistic regression analysis, using age and sex as covariates, revealed no association of the single-nucleotide polymorphism with overweight (BMI ≥ 25) between rs7566605 genotypes in the Japanese cohort (CC vs. CG/GG, odds ratio = 1.18; 95% confidence interval = 0.84 to 1.65, $p = 0.333$; CC vs. GG, odds ratio = 1.19, 95% confidence interval = 0.84 to 1.69, $p = 0.325$). No significant associations were observed between polymorphism and glucose or insulin levels. These results suggested no association of the rs7566605 variant with overweight in Japanese people.

Key words: glucose metabolism, genotype, BMI, body weight, insulin resistance

Recently, Herbert et al. (1) demonstrated that a common genetic single-nucleotide polymorphism (rs7566605) upstream of the transcription start site for insulin-induced gene 2 was associated with obesity in 694 individuals of the National Heart, Lung, and Blood Institute-Framingham Heart Study. Analysis suggests that rs7566605 CC homozygotes (C allele frequency, 0.37) have higher BMI levels than individuals with GC or GG genotypes, regardless of sex or age. This finding was replicated in four of five populations studied. A meta-analysis of all four case-control samples showed that CC homozygosity was also significantly associated with obesity (BMI ≥ 30 kg/m²), with an odds ratio (OR)¹ of 1.22 [95% confidence interval (CI), 1.05 to 1.42]. However, more recent studies do not support the association of the rs7566605 polymorphism with obesity for the different samples (2–4). There may be many reasons that the association is not seen in these studies, including those related to study design, underlying genetic heterogeneity of populations, and different environmental exposures. However, most of the samples studied were of European/European origin or African Americans. We tested whether this variant is also linked to obesity/overweight among Asian populations. Our sample consisted of 2233 randomly selected, community dwelling, middle-age and older Japanese people.

The genotype frequencies for rs7566605 polymorphism were: GG, 0.465; GC, 0.432; and CC, 0.104 (C allele frequency, 0.32), which are similar frequencies to those reported by Herbert et al. (1) (Table 1). These frequencies are consistent with those expected under Hardy-Weinberg equilibrium (1 *d/f*). There were no significant differences in the genotype distributions of rs7566605 polymorphism between men and women or among the different age groups (Table 1). Table 2 shows the mean (standard error) of BMI, other anthropometric variables, and glucose metabolic variables tested in the GG/GC and CC genotypes. There were

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¹ Nonstandard abbreviations: OR, odds ratio; CI, confidence interval; NILS, National Institute for Longevity Sciences; LSA, Longitudinal Study of Aging.

Table 1. Distribution of rs7566605 genotypes

	n	CC		GC		GG		GC/GG	
		n	%	n	%	n	%	n	%
Total	2233	233	10.4	962	43.1	1038	46.5	2000	89.6
Men*	1128	121	10.7	470	41.7	537	47.6	1007	89.3
Women	1105	112	10.1	492	44.5	501	45.3	993	89.9
Age (yrs)†									
40 to 49	563	61	10.8	224	39.8	278	49.4	502	89.2
50 to 59	556	56	10.1	265	47.7	235	42.3	500	89.9
60 to 69	561	64	11.4	241	43.0	256	45.6	497	88.6
70 to 79	553	52	9.4	232	42.0	269	48.6	501	90.6

* CC, GC, GG, men vs. women, $\chi^2 = 1.863$, $p = 0.394$; CC, GC/GG, men vs. women, $\chi^2 = 0.209$, $p = 0.648$.

† CC, GC, GG, age groups, $\chi^2 = 9.306$, $p = 0.157$; CC, GC/GG, age groups, $\chi^2 = 1.373$, $p = 0.712$.

no differences in BMI levels between these genotypes. In addition, no significant differences in waist and hip circumferences, waist-to-hip-ratio, and fat mass were observed between these genotypes in either gender (Table 2). No significant association was observed between these genotypes and fasting glucose, insulin, hemoglobin A_{1c}, or homeostasis model assessment for insulin resistance levels in men or women (Table 2). The rs7566605 genotypes showed similar allele frequencies in diabetic individuals and in non-diabetic controls (data not shown).

In our cohort, only a small number of participants had a BMI ≥ 30 kg/m² (0.97% in men, 3.37% in women). When logistic regression was performed to calculate the OR for the CC genotype compared with the CG/GG genotypes or the GG genotype, defining overweight as a BMI ≥ 25 kg/m² (23.6% in men, 22.0% in women), using age and sex as covariates, the CC genotype showed no association of the single-nucleotide polymorphism with overweight (vs. CG/GG, OR = 1.18, 95% CI, 0.84 to 1.65, $p = 0.333$; vs. GG, OR = 1.19, 95% CI, 0.84 to 1.69, $p = 0.325$). The CC/CG

Table 2. Anthropometric and glucose metabolic variables according to rs7566605 genotypes

	Men						Women					
	n	CC		GG/GC		p	n	CC		GG/GC		p
		Mean	SE	Mean	SE			Mean	SE	Mean	SE	
Weight (kg)	1127	62.0	0.8	62.1	0.3	0.804	1104	52.8	0.8	52.4	0.3	0.612
Height (cm)	1127	164.3	0.5	164.6	0.2	0.695	1104	151.9	0.5	151.2	0.2	0.176
BMI (kg/m ²)	1127	22.9	0.3	22.9	0.1	0.820	1104	22.8	0.3	22.9	0.1	0.792
Waist circumference (cm)	1127	82.0	0.8	82.4	0.3	0.585	1104	75.2	0.9	75.1	0.3	0.969
Hip circumference (cm)	1127	90.9	0.4	91.1	0.2	0.622	1104	90.6	0.5	90.6	0.2	0.985
Waist-to-hip ratio	1127	0.9	0.01	0.9	0.002	0.641	1104	0.8	0.01	0.8	0.002	0.877
Fat mass (kg)	1125	21.0	0.4	21.4	0.1	0.335	1096	31.4	0.5	31.5	0.2	0.864
Glucose (mM)*	1056	5.8	0.1	5.7	0.03	0.233	1049	5.4	0.1	5.5	0.03	0.304
Insulin (μ U/mL)*	1071	8.0	0.5	8.2	0.2	0.691	1069	7.8	0.5	8.4	0.2	0.210
Hemoglobin A _{1c} (%)*	1055	5.3	0.1	5.2	0.02	0.373	1048	5.1	0.05	5.2	0.02	0.252
HOMA-IR*	1055	2.2	0.2	2.2	0.1	0.959	1048	1.9	0.2	2.1	0.1	0.190

SE, standard error; HOMA-IR, homeostasis model assessment of insulin resistance.

* Analysis of subjects who were not on oral hypoglycemic agents or insulin. Data were adjusted for age.

genotype also showed no significant association of the single-nucleotide polymorphism with overweight under a recessive model (vs. GG, OR = 1.05, 95% CI, 0.86 to 1.28, $p = 0.614$).

These results suggested no association of the rs7566605 variant with overweight in Japanese people. An ethnic difference may have contributed to the lack of this association. Another possibility is that the lower BMI levels and fewer obese individuals in our cohort may have affected our results. It is possible that due to the low number of obese individuals in our population, we lacked sufficient power to attempt replication of the previously reported association between rs7566605 and obesity in this study. In fact, it has been reported that excluding the upper quartile from the analysis in the Kooperative Gesundheitsforschung in der Region Augsburg (KORA) population in the study by Herbert et al. (1) eliminated the evidence for association of rs7566605 CC genotype with obesity, indicating that an association with obesity is strongest in those who are more obese. However, similar genotype frequency for this variant between our cohort and others may indicate that environmental influences can overcome the genetic influence on the anthropometric measurement.

Research Methods and Procedures

The present study consisted of a cross-sectional analysis of 1105 women and 1128 men who participated in the first wave of examinations in the National Institute for Longevity Sciences (NILS)-Longitudinal Study of Aging (LSA) from April of 1998 to March of 2000. The subjects of the NILS-LSA were male and female residents, 40 to 79 years old. The population of Obu city and Higashiura town in the Aichi prefecture in central Japan was stratified by both age and gender and randomly selected from resident registrations in cooperation with the local governments. The number of men and women was to be the same to test gender difference. Age at baseline was to be 40 to 79 years, and the number of participants in each decade (40s, 50s, 60s, 70s) was to be the same. The examinations include various areas of gerontology and geriatrics such as medical examinations, anthropometry, body composition, physical functions, physical activities, psychological assessments, nutritional analysis, and molecular epidemiology. The subjects will be followed up every 2 years. The details of the NILS-LSA have been described elsewhere (5,6). Randomly selected men and women were invited by mail to attend an explanatory meeting. At that meeting, the procedures for each examination and the follow-up schedule were fully explained. Written, informed consent for the entire procedure was obtained from each participant. The study was approved by the Ethics Committee of the NILS.

Anthropometric Variables

Body weight was measured to the nearest 0.01 kg using a digital scale, height was measured to the nearest 0.1 cm

using a wall-mounted stadiometer, and BMI was calculated as weight (kilograms) divided by height squared (meters squared). Waist circumference and waist-to-hip ratio were used as the indices for body fat distribution in this study. Waist-to-hip ratio was calculated as the ratio of waist circumference measured at the midpoint between the anterior superior iliac crest and the lowest rib-to-hip circumference. Whole-body fat mass, assessed by DXA (QDR-4500; Hologic, Madison, WI), was used as an index for determining body composition.

Biochemical Assays of Blood

An antecubital blood sample was drawn from each subject after an overnight fast. Fasting plasma glucose was assayed by the glucose oxidase method (7). Plasma insulin was measured by radioimmunoassay (8). Coefficients of variation of glucose and insulin were 16.3% and 64.3%, respectively. The homeostasis model assessment for insulin resistance was calculated as fasting serum insulin (microunits per milliliter) \times fasting plasma glucose (millimolar)/22.5 (9).

Determination of rs7566605 Genotypes

Genotypes were determined using a fluorescence-based allele-specific DNA primer assay system (Toyobo Tsuruga Gene Analysis, Tsuruga, Japan). The polymorphic regions of rs7566605 were amplified by polymerase chain reaction with allele-specific sense primers labeled at the 5' end with either fluorescein isothiocyanate (5'-TCATTGCAATAGC-CACTGCCAAGTAC-3') or Texas red (5'-GGATATTGATCGTGGTCCTTTA-3') as allele-specific hybridization probe and with an antisense primer labeled at the 5'-end with biotin (5'-AAAAACTGAAAACCACCCTGGTACAGAC-3'). The reaction mixtures (25 μ L) contained 20 ng of DNA, 5 pmol of each primer, 0.2 mM deoxynucleoside triphosphate, 2.0 mM MgCl₂, and 1 U of rTaq DNA polymerase (Toyobo Co., Ltd.) in polymerase buffer. The amplification protocol consisted of initial denaturation at 95 °C for 5 minutes, followed by 45 cycles of denaturation at 95 °C for 30 seconds, annealing at 65 °C for 30 seconds, and extension at 72 °C for 30 seconds; a final extension was conducted at 72 °C for 2 minutes. Our genotyping error rate was ~0.1%.

Data Analysis

Quantitative data adjusted for age were compared between the two groups by unpaired Student's *t* test. Allele frequencies were estimated by the gene counting method, and the χ^2 test was used to identify any significant departure from Hardy-Weinberg equilibrium. Logistic regression was performed to calculate the OR for the CC allele genotype compared with CG/GG genotypes or the GG genotype, defining overweight as BMI ≥ 25 kg/m², using age and sex as covariates. In this study, the significant difference in BMI

by genotype should be $>0.76 \text{ kg/m}^2$ in men and $>0.92 \text{ kg/m}^2$ in women with a power ($1 - \beta$) of 0.8 and an α of 0.05. In the analyses to examine the association between genotypes and glucose metabolisms, participants who were being treated with oral hypoglycemic agents or insulin were excluded. The general linear model was applied to control for age. In the model, each quantitative variable was the dependent variable, and age and genotype were the independent variables. Least square means of the dependent variable by genotype were compared and tested by Student's *t* test. A *p* value <0.05 was considered to be statistically significant. The data were analyzed with SAS, release 8.2 (SAS Institute, Inc., Cary, NC).

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