and a large population is necessary to have enough cases for analysis. One way to overcome this disadvantage is to use a surrogate condition such as SBI for the cases.

Asymptomatic brain infarction is reported to be common in the elderly population. Although neurologically asymptomatic, MRI-proven abnormal findings have been shown to be associated with several disorders.[16,17,22–25] Accordingly, it is conceivable that the subjects with SBI might not have been completely free of symptoms in the present study. In our previous studies, SBI was shown to be associated with hypertensive end-organ damage[24] and abnormal diurnal changes in blood pressure.[17,25] Although we did not analyze cognitive function in the present study, an association between impaired cognitive function and SBI and white matter lesions has also been reported.[14,22] In several studies, the MTHFR TT genotype has also been demonstrated to be a risk factor for dementia,[26,27] although conflicting results have also been reported.[28,29] These findings suggest a possible association between the MTHFR TT genotype and cognitive impairment in the general population. However, this issue needs to be confirmed in a large community population.

Although the origin of the TT variant is not known, the high prevalence of this polymorphism in most populations could indicate that the TT variant might represent an ancestral genetic adaptation to living.[12] Enhanced homeostasis, cell proliferation, and tissue repair have been postulated as underlying mechanisms.[12] The MTHFR TT variant shows reduced enzymatic activity, resulting in an increase in Hcy concentration. It has been reported that hyperhomocysteinemia is a possible causal factor in free radical generation during the acute phase of thrombotic cerebrovascular stroke.[30] Recently, it has also been reported that the MTHFR TT genotype was associated with reduced superoxide dismutase activity.[31] Because DNA can be damaged in oxidative stress such as ischemia-reperfusion injury in the brain, DNA repair after oxidative stress could also be altered by the TT mutation. Furthermore, it has been reported that errors in DNA repair caused by oxidative stress occur preferentially at GC sequences in the brain,[32,33] suggesting that the TT mutation could be a hallmark of oxidative stress in the brain. These findings suggest that damage caused by oxidative stress such as reperfusion could be enhanced in subjects with the MTHFR TT mutation, which may account for their higher prevalence of SBI. However, this hypothesis requires further study.

In summary, in a large, randomly selected, community-based population, the MTHFR TT genotype was an independent risk factor for both SBI and white matter lesions. Because the prevalence of SBI is relatively high, an interventional approach could be useful in reducing the future risk of developing symptomatic stroke and cognitive decline.

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Association of Polymorphisms of Interleukin-6, Osteocalcin, and Vitamin D Receptor Genes, Alone or in Combination, with Bone Mineral Density in Community-Dwelling Japanese Women and Men

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We examined whether the −634C→G, 298C→T, and 2C→T polymorphisms of the IL-6, osteocalcin, and vitamin D receptor (VDR) genes, respectively, were associated, alone or in combination, with bone mineral density (BMD) in community-dwelling Japanese women (between 1108 and 1113) or men (between 1116 and 1130) aged 40–79 yr. The −634C→G polymorphism of the IL-6 gene and the 298C→T polymorphism of the osteocalcin gene were associated with BMD in postmenopausal women, with the respective GG and TT genotypes representing risk factors for reduced bone mass. IL-6 and osteocalcin genotypes showed additive effects on BMD for postmenopausal women. The 2C→T polymorphism of the VDR gene was associated with BMD in men, with the CT genotype contributing to reduced BMD. These results suggest that the IL-6 and osteocalcin genes are susceptibility loci for reduced BMD in postmenopausal women and that the VDR gene constitutes such a locus in men. The combined IL-6 and osteocalcin genotypes may prove informative for the assessment of osteoporosis in women. (*J Clin Endocrinol Metab* 88: 3372–3378, 2003)

OSTEOPOROSIS IS CHARACTERIZED by a decrease in bone mineral density (BMD) and a deterioration in the microarchitecture of bone, both of which result in an increased susceptibility to fractures (1). Although several environmental factors, such as diet and physical exercise, influence BMD, a genetic contribution to the etiology of osteoporosis has been recognized (2). Genetic linkage studies (3, 4) and candidate gene association studies (5, 6) have implicated several loci and candidate genes in the regulation of bone mass and the pathogenesis of osteoporotic fractures. Such candidate genes also include those for IL-6, osteocalcin, and the vitamin D receptor (VDR).

IL-6 is a multifunctional cytokine that is important in the development of postmenopausal osteoporosis (7). Sibling-pair analysis has provided evidence of linkage between the IL-6 gene locus and reduced BMD in postmenopausal Japanese women (8). Three polymorphisms of the IL-6 gene have been identified in Japanese, among which a C→G substitution at nucleotide −634 in the promoter region has been associated with radial BMD (9). A variable number of tandem repeats polymorphism in the 3′ flanking region of the IL-6 gene has also been associated with BMD in postmenopausal Caucasian women (10).

Osteocalcin is an extracellular matrix protein that is abundant in bone. Characterization of osteocalcin-deficient mice demonstrated that this protein functions as a negative regulator of bone formation (11). A C→T polymorphism at nucleotide 298 in the promoter region of the osteocalcin gene was identified and shown to be associated with BMD in a small Japanese population (12).

A *BsmI* restriction fragment length polymorphism (RFLP) of the VDR gene was shown to be associated with BMD in Australian women (13). A 2C→T polymorphism (ACG→ATG) at the translation initiation site of the VDR gene has also been associated with BMD in postmenopausal Mexican-American (14) and Japanese (15) women, but not in premenopausal French women (16). The 2C→T polymorphism was also associated with BMD in men (17, 18) and with calcium absorption and BMD in girls and boys of various ethnic ancestries (19).

Although the association of these various single nucleotide polymorphisms (SNPs) with BMD suggests that the IL-6, osteocalcin, and VDR genes might be susceptibility loci for osteoporosis in women, large-scale population-based studies identifying the association of these SNPs with BMD simultaneously are required to clarify their roles in determination of this parameter. In addition, the relation of these SNPs to BMD in men has not been definitively identified. We have now examined whether the −634C→G SNP of the IL-6 gene, the 298C→T SNP of the osteocalcin gene, and the 2C→T SNP of the VDR gene are associated with BMD in women or men in a large-scale population-based study.

Abbreviations: BMD, Bone mineral density; BMI, body mass index; DXA, dual-energy X-ray absorptiometry; PCR, polymerase chain reaction; pQCT, peripheral quantitative computed tomography; RFLP, restriction fragment length polymorphism; SNP, single nucleotide polymorphism; VDR, vitamin D receptor.

Subjects and Methods

Study population

The National Institute for Longevity Sciences–Longitudinal Study of Aging (NILS-LSA) is a population-based prospective cohort study of

aging and age-related diseases (20). The subjects of the NILS-LSA are stratified by both age and gender, and are randomly selected from resident registrations in the city of Obu and town of Higashiura in central Japan. 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 are similar, and age at the baseline is 40–79 yr, with similar numbers of participants in each decade (40s, 50s, 60s, and 70s). The subjects will be followed up every 2 yr. All participants are subjected at a special center to a detailed examination, which includes not only medical evaluation but also assessment of exercise physiology, body composition, nutrition, and psychology. We have examined the association of BMD at various sites with the −634C→G SNP of the IL-6 gene in 2239 participants (1113 women, 1126 men), with the 298C→T SNP of the osteocalcin gene in 2224 participants (1108 women, 1116 men), and with the 2C→T SNP of the VDR gene in 2238 participants (1108 women, 1130 men). The study protocol was approved by the Committee on Ethics of Human Research of National Chubu Hospital and the NILS, and written informed consent was obtained from each subject.

Measurement of BMD

BMD of the nondominant radius was measured by peripheral quantitative computed tomography (pQCT) (Desiscan 1000, Scanco Medical, Bassersdorf, Switzerland), with the forearm positioned in a cast during measurement. For measurement of BMD of the distal radius, the examination was initiated at a position 6.0 mm from the distal end of the radius and progressed proximally with 10 tomographs (slice thickness, 1 mm; interslice distance, 0.5 mm), and the average BMD was calculated. D50 represented distal radius BMD for the inner 50% of the cross-sectional area, comprising mostly cancellous bone, and D100 represented that for the entire cross-sectional area, including both cancellous and cortical bone. BMD for the proximal radius was measured at the diaphysis; the examination was initiated at a site 27.5 mm from the last slice of the distal radius and proceeded proximally with six tomographs, and the average BMD was calculated. P100 represented proximal radius BMD for the entire cross-sectional area, consisting mostly of cortical bone. BMD for total body, lumbar spine (L2–L4), right femoral neck, right trochanter, and right Ward's triangle was measured by dual-energy x-ray absorptiometry (DXA) (QDR 4500; Hologic, Inc., Bedford, MA). The coefficients of variance 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), 1.0% (trochanter), and 2.5% (Ward's triangle).

Determination of IL-6, osteocalcin, and VDR genotypes

IL-6, osteocalcin, and VDR genotypes were determined with a fluorescence- or colorimetry-based allele-specific DNA primer assay system (Toyobo Gene Analysis, Tsuruga, Japan). For determination of IL-6 genotype, the polymorphic region of the gene was amplified by the polymerase chain reaction (PCR) with allele-specific sense primers labeled at the 5′ end either with fluorescein isothiocyanate (5′-GGCAGTTCTACAACAGCXCC-3′) or with Texas red (5′-GCAGTTCTACAACAGCXGC-3′) and an antisense primer labeled at the 5′ end with biotin (5′-CTGTGTTCTGGCTCTCCCTG-3′). For determination of osteocalcin or VDR genotype, the polymorphic region of the gene was amplified by PCR with allele-specific sense primers (5′-CAGCTCCCAACCACAATATCCXTT-3′ or 5′-CAGCTCCCAACCACAATATCCXCT-3′ for osteocalcin genotype, and 5′-CTTGCTGTTCTTACAGGGXTG-3′ or 5′-CTTGCTGTTCTTACAGGGXCG-3′ for VDR genotype) and an antisense primer labeled at the 5′ end with biotin (5′-GTGTGAGGGCTCTCATGGTGT-3′ for osteocalcin genotype, 5′-AAGTGCTGGCCGCCATTG-3′ for VDR genotype). The reaction mixture (25 μl) contained 20 ng of DNA, 5 pmol of each primer, 0.2 mmol/liter of each deoxynucleoside triphosphate, 1–4 mmol/liter $MgCl_2$, and 1 U DNA polymerase (rTaq or KODplus; Toyobo, Osaka, Japan) in the respective DNA polymerase buffer. The amplification protocol comprised initial denaturation at 95 C for 5 min; 35 (IL-6 genotype), 45 (osteocalcin genotype), or 40 (VDR genotype) cycles of denaturation at 95 C for 30 sec, annealing at 55–65 C for 30 sec, and extension at 72 C for 30 sec; and a final extension at 72 C for 2 min.

For determination of IL-6 genotype, 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 supernatants were then collected from each well, transferred to the wells of a 96-well plate containing 0.01 mol/liter NaOH, and 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.

For determination of osteocalcin or VDR genotype, amplified DNA was denatured with 0.3 mol/liter NaOH and then subjected to hybridization at 37 C for 30 min in hybridization buffer containing 35–40% formamide with allele-specific capture probes (5′-CACAATATCCX-TTGGGGTTT-3′ or 5′-CACAATATCCXCTGGGGTT-3′ for osteocalcin genotype, and 5′-TACAGGGXTGGAGGCAATG-3′ or 5′-TACAGGGX-CGGAGGCAATG-3′ for VDR genotype) fixed to the bottom of the wells of a 96-well plate. After thorough washing of the wells, alkaline phosphatase-conjugated streptavidin was added to each, and the plate was incubated at 37 C for 15 min with agitation. The wells were washed again, and, after the addition of a solution containing 0.8 mmol/liter 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium (monosodium salt) and 0.4 mmol/liter 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt, absorbance at 450 nm was measured.

To confirm the accuracy of genotyping by this method, we selected 50 DNA samples at random and subjected them to PCR-RFLP analysis or direct DNA sequencing of PCR products. In each instance, the genotype determined by the allele-specific DNA primer assay system was identical to that determined by PCR-RFLP analysis or DNA sequencing.

Statistical analysis

Quantitative data were compared by one-way ANOVA and the Tukey-Kramer *post hoc* test. BMD values were analyzed with adjustment for age and body mass index (BMI) by the least-squares method in a general linear model. Qualitative data were analyzed by the χ^2 test. Allele frequencies were estimated by the gene-counting method, and the χ^2 test was used to identify significant departure from Hardy-Weinberg equilibrium. $P \leq 0.05$ was considered statistically significant.

Results

The relation of the −634C→G SNP in the promoter region of the IL-6 gene to BMD was examined. The distributions of IL-6 genotypes with regard to this SNP in women (Table 1) and in men (Table 2) were in Hardy-Weinberg equilibrium. Age and BMI did not differ among IL-6 genotypes in women (Table 1). For men, age did not differ among IL-6 genotypes, but BMI was significantly greater for those with the GG genotype than for those with the CG genotype (Table 2). Among all women, BMD for the total body or lumbar spine was smaller in those with the GG genotype than in those with the CC or CG genotypes, with adjustment for age and BMI (Table 1). To examine the influence of menopause on the relation between −634C→G genotype and BMD, we analyzed BMD and other characteristics for premenopausal and postmenopausal women independently. Given that the number of perimenopausal women was small (n = 17), these subjects were excluded from the analysis. For premenopausal women, BMD was not associated with IL-6 genotype. In contrast, for postmenopausal women, BMD for the distal radius (D50 and D100), total body, lumbar spine, femoral neck, trochanter, or Ward's triangle was significantly lower in those with the GG genotype than in those with the CC or CG genotypes (Table 1). The differences in BMD between the CC and GG genotypes (expressed as a percentage of the corresponding larger value) in postmenopausal women were 13.7% (D50), 7.2% (D100), 4.3% (total body), 8.4% (lumbar spine), 5.3% (femoral neck), 5.2% (trochanter), and 8.6%

TABLE 1. BMD and other characteristics of women according to IL-6 genotype

Characteristic	Total (n = 1113)			Premenopausal (n = 279)			Postmenopausal (n = 817)		
	CC	CG	GG	CC	CG	GG	CC	CG	GG
Number (%)	638 (57.3)	406 (36.5)	69 (6.2)	170 (60.9)	91 (32.6)	18 (6.5)	455 (55.7)	311 (38.1)	51 (6.2)
Age (yr)	59.1 ± 0.4	59.4 ± 0.5	60.4 ± 1.3	46.4 ± 0.4	45.8 ± 0.5	46.2 ± 1.1	64.1 ± 0.4	63.5 ± 0.5	65.4 ± 1.2
BMI (kg/m^2)	22.9 ± 0.1	22.9 ± 0.2	23.1 ± 0.4	22.6 ± 0.2	23.1 ± 0.3	22.6 ± 0.8	23.0 ± 0.2	22.8 ± 0.2	23.2 ± 0.5
BMD values measured by pQCT (mg/cm^3)									
D50	185.5 ± 2.5	184.3 ± 3.1	171.7 ± 7.6	244.9 ± 4.2	244.8 ± 5.8	255.8 ± 13.5	165.1 ± 3.0	164.5 ± 3.6	142.4 ± 9.0[a,b]
D100	486.2 ± 3.6	483.8 ± 4.5	465.2 ± 11.0	607.8 ± 6.0	598.8 ± 8.3	616.5 ± 19.3	444.0 ± 4.3	446.1 ± 5.2	412.2 ± 13.0[a,b]
P100	1153.1 ± 5.8	1148.4 ± 7.3	1126.7 ± 17.8	1360.1 ± 9.2	1354.8 ± 12.7	1376.5 ± 29.5	1081.7 ± 7.1	1080.4 ± 8.6	1039.8 ± 21.5
BMD values measured by DXA (g/cm^2)									
Total body	0.968 ± 0.003	0.961 ± 0.004	0.938 ± 0.010[a]	1.093 ± 0.006	1.098 ± 0.009	1.089 ± 0.020	0.924 ± 0.004	0.916 ± 0.005	0.884 ± 0.012[b,c]
L2–L4	0.869 ± 0.005	0.867 ± 0.006	0.807 ± 0.015[c,d]	1.026 ± 0.009	1.036 ± 0.012	0.974 ± 0.027	0.813 ± 0.006	0.813 ± 0.007	0.745 ± 0.018[e,f]
Femoral neck	0.678 ± 0.003	0.679 ± 0.004	0.655 ± 0.010	0.770 ± 0.007	0.775 ± 0.010	0.777 ± 0.022	0.646 ± 0.004	0.646 ± 0.005	0.612 ± 0.012[a,b]
Trochanter	0.572 ± 0.003	0.571 ± 0.004	0.554 ± 0.010	0.654 ± 0.006	0.665 ± 0.009	0.665 ± 0.020	0.543 ± 0.004	0.539 ± 0.005	0.515 ± 0.012[a]
Ward's triangle	0.508 ± 0.005	0.507 ± 0.006	0.480 ± 0.014	0.657 ± 0.009	0.665 ± 0.013	0.658 ± 0.028	0.456 ± 0.005	0.453 ± 0.007	0.417 ± 0.016[a]

Data are expressed as means ± SE. BMD values were adjusted for age and BMI.
[a] $P \leq 0.05$ *vs.* CC; [b] $P \leq 0.05$ *vs.* CG; [c] $P \leq 0.005$ *vs.* CC; [d] $P \leq 0.001$ *vs.* CG; [e] $P \leq 0.001$ *vs.* CC; [f] $P \leq 0.005$ *vs.* CG.

TABLE 2. BMD and other characteristics of men (n = 1126) according to IL-6 genotype

Characteristic	CC	CG	GG
Number (%)	664 (59.0)	392 (34.8)	70 (6.2)
Age (yr)	59.7 ± 0.4	58.7 ± 0.6	57.2 ± 1.3
BMI (kg/m^2)	23.0 ± 0.1	22.7 ± 0.1	23.7 ± 0.3[a]
BMD values measured by pQCT (mg/cm^3)			
D50	268.4 ± 2.6	263.6 ± 3.4	265.6 ± 7.8
D100	543.4 ± 3.6	537.7 ± 4.6	534.6 ± 10.9
P100	1189.6 ± 5.5	1180.5 ± 7.2	1172.9 ± 16.7
BMD values measured by DXA (g/cm^2)			
Total body	1.088 ± 0.004	1.088 ± 0.005	1.077 ± 0.011
L2–L4	0.985 ± 0.006	0.982 ± 0.008	0.966 ± 0.018
Femoral neck	0.756 ± 0.004	0.752 ± 0.005	0.736 ± 0.012
Trochanter	0.670 ± 0.004	0.668 ± 0.005	0.652 ± 0.012
Ward's triangle	0.556 ± 0.005	0.549 ± 0.006	0.541 ± 0.014

Data are expressed as means ± SE. BMD values were adjusted for age and BMI.
[a] $P \leq 0.05$ *vs.* CG.

(Ward's triangle). No significant difference in BMD among IL-6 genotypes was detected for men (Table 2).

The relation of the 298T→C SNP in the promoter region of the osteocalcin gene to BMD was examined. The distributions of osteocalcin genotypes with regard to this SNP were in Hardy-Weinberg equilibrium in women (Table 3) and in men (Table 4). Age did not differ among osteocalcin genotypes for all women or for premenopausal women, but it was greater for postmenopausal women with the TT genotype than for those with the CC genotype (Table 3). For men, age did not differ among osteocalcin genotypes (Table 4). BMI did not differ among osteocalcin genotypes in all, premenopausal, or postmenopausal women or in men. Among women, BMD for the total body, lumbar spine, or Ward's triangle was greater in those with the CC genotype than in those with the TT or CT genotypes (Table 3). For premenopausal women, BMD was not associated with osteocalcin genotype. In contrast, for postmenopausal women, BMD for the total body, lumbar spine, femoral neck, trochanter, or Ward's triangle was greater in those with the CC genotype than in those with the TT or CT genotypes. The differences in BMD between the CC and TT genotypes in postmenopausal women were 4.4% (total body), 8.3% (lumbar spine), 5.3% (femoral neck), 6.5% (trochanter), and 14.7% (Ward's triangle). For men, BMD did not differ significantly among osteocalcin genotypes (Table 4).

The relation of the 2C→T SNP in the translation initiation codon of the VDR gene to BMD was examined. The distributions of VDR genotypes with regard to this SNP in women (Table 5) and in men (Table 6) were in Hardy-Weinberg equilibrium. Age and BMI did not differ among VDR genotypes in women (Table 5) or in men (Table 6). BMD did not differ among VDR genotypes for all women or for premenopausal or postmenopausal women (Table 5, data not shown). Among men, BMD for the distal radius (D50), femoral neck, or Ward's triangle was smaller in those with the CT genotype than in those with the CC genotype (Table 6). The differences in BMD between the CC and CT genotypes in men were 3.6% (D50), 2.1% (femoral neck), and 3.4% (Ward's triangle).

The effects of IL-6, osteocalcin, and VDR genotypes as well as of other characteristics on BMD at various sites were analyzed by multivariate regression analysis (Table 7). For

TABLE 3. BMD and other characteristics of women according to osteocalcin genotype

Characteristic	Total (n = 1108)			Premenopausal (n = 278)			Postmenopausal (n = 813)		
	CC	CT	TT	CC	CT	TT	CC	CT	TT
Number (%)	50 (4.5)	357 (32.2)	701 (63.3)	12 (4.3)	86 (30.9)	180 (64.8)	38 (4.7)	265 (32.6)	510 (62.7)
Age (yr)	57.5 ± 1.5	59.0 ± 0.6	59.5 ± 0.4	46.7 ± 1.3	46.1 ± 0.5	46.2 ± 0.3	60.9 ± 1.4	63.3 ± 0.6	64.4 ± 0.4[a]
BMI (kg/m^2)	23.7 ± 0.5	22.9 ± 0.2	22.8 ± 0.1	24.1 ± 0.9	23.0 ± 0.3	22.5 ± 0.2	23.5 ± 0.5	22.8 ± 0.2	23.0 ± 0.1
BMD values measured by pQCT (mg/cm^3)									
D50	195.8 ± 8.7	189.5 ± 3.3	181.0 ± 2.4	240.8 ± 16.4	252.5 ± 6.0	241.9 ± 4.1	182.3 ± 10.1	168.5 ± 3.9	159.9 ± 2.8
D100	494.5 ± 12.5	489.2 ± 4.8	481.4 ± 3.4	620.7 ± 23.2	613.5 ± 8.5	601.0 ± 5.9	456.0 ± 14.6	447.7 ± 5.6	440.1 ± 4.1
P100	1175.4 ± 20.3	1152.0 ± 7.7	1148.9 ± 5.5	1404.2 ± 35.7	1359.7 ± 13.0	1358.5 ± 9.0	1103.3 ± 24.1	1082.2 ± 9.3	1076.9 ± 6.7
BMD values measured by DXA (g/cm^2)									
Total body	0.993 ± 0.012	0.968 ± 0.005	0.959 ± 0.003[b]	1.109 ± 0.024	1.099 ± 0.009	1.090 ± 0.006	0.955 ± 0.014	0.924 ± 0.005	0.913 ± 0.004[b]
L2–L4	0.910 ± 0.018	0.867 ± 0.007	0.860 ± 0.005[a]	1.029 ± 0.034	1.027 ± 0.013	1.024 ± 0.009	0.875 ± 0.021	0.814 ± 0.008[a]	0.802 ± 0.006[b]
Femoral neck	0.694 ± 0.012	0.681 ± 0.005	0.674 ± 0.003	0.745 ± 0.027	0.781 ± 0.010	0.769 ± 0.007	0.676 ± 0.014	0.648 ± 0.005	0.640 ± 0.004[a]
Trochanter	0.588 ± 0.012	0.574 ± 0.004	0.567 ± 0.003	0.626 ± 0.024	0.665 ± 0.009	0.656 ± 0.006	0.573 ± 0.013	0.543 ± 0.005	0.536 ± 0.004[a]
Ward's triangle	0.554 ± 0.016	0.510 ± 0.006[a]	0.501 ± 0.004[c]	0.639 ± 0.035	0.672 ± 0.013	0.654 ± 0.009	0.524 ± 0.019	0.454 ± 0.007[c]	0.447 ± 0.005[d]

Data are expressed as means ± SE. BMD values were adjusted for age and BMI.
[a] $P \leq 0.05$ *vs.* CC; [b] $P \leq 0.01$ *vs.* CC; [c] $P \leq 0.005$ *vs.* CC; [d] $P \leq 0.001$ *vs.* CC.

TABLE 4. BMD and other characteristics of men (n = 1116) according to osteocalcin genotype

Characteristic	CC	CT	TT
n (%)	56 (5.0)	380 (34.1)	680 (60.9)
Age (yr)	60.4 ± 1.5	58.8 ± 0.6	59.2 ± 0.4
BMI (kg/m^2)	22.3 ± 0.4	23.0 ± 0.1	22.9 ± 0.1
BMD values measured by pQCT (mg/cm^3)			
D50	260.6 ± 8.9	272.3 ± 3.4	264.0 ± 2.5
D100	539.6 ± 12.3	545.9 ± 4.8	538.5 ± 3.5
P100	1208.6 ± 18.9	1187.3 ± 7.3	1182.1 ± 5.4
BMD values measured by DXA (g/cm^2)			
Total body	1.096 ± 0.012	1.095 ± 0.005	1.082 ± 0.003
L2–L4	0.968 ± 0.020	0.992 ± 0.008	0.978 ± 0.006
Femoral neck	0.743 ± 0.013	0.761 ± 0.005	0.749 ± 0.004
Trochanter	0.651 ± 0.013	0.673 ± 0.005	0.666 ± 0.004
Ward's triangle	0.538 ± 0.016	0.557 ± 0.006	0.551 ± 0.005

Data are expressed as means ± SE. BMD values were adjusted for age and BMI.

TABLE 5. BMD and other characteristics of women (n = 1108) according to VDR genotype

Characteristic	CC	CT	TT
Number (%)	457 (41.3)	504 (45.5)	147 (13.3)
Age (yr)	59.9 ± 0.5	58.7 ± 0.5	59.3 ± 0.9
BMI (kg/m^2)	22.8 ± 0.2	23.1 ± 0.1	22.7 ± 0.3
BMD values measured by pQCT (mg/cm^3)			
D50	184.3 ± 2.9	184.2 ± 2.8	190.0 ± 5.1
D100	486.2 ± 4.2	484.3 ± 4.0	487.4 ± 7.4
P100	1153.5 ± 6.8	1151.1 ± 6.5	1159.9 ± 12.0
BMD values measured by DXA (g/cm^2)			
Total body	0.966 ± 0.004	0.965 ± 0.004	0.965 ± 0.007
L2–L4	0.871 ± 0.006	0.861 ± 0.006	0.866 ± 0.011
Femoral neck	0.678 ± 0.004	0.681 ± 0.004	0.670 ± 0.007
Trochanter	0.570 ± 0.004	0.573 ± 0.004	0.566 ± 0.007
Ward's triangle	0.507 ± 0.005	0.509 ± 0.005	0.500 ± 0.010

Data are expressed as means ± SE. BMD values were adjusted for age and BMI.

women, IL-6 genotype significantly affected BMD for the total body and lumbar spine, and osteocalcin genotype significantly contributed to BMD for the distal radius (D50), total body, lumbar spine, femoral neck, trochanter, and Ward's triangle. For men, osteocalcin genotype significantly influenced BMD for the total body, and VDR genotype significantly contributed to BMD for the distal radius (D50), femoral neck, trochanter, and Ward's triangle.

To determine whether the −634C→G SNP of the IL-6 gene and the 298C→T SNP of the osteocalcin gene exert an additive effect on BMD in women, we examined the association between the combined genotype and BMD. The distribution of the combined IL-6 and osteocalcin genotypes for women is shown in Table 8. The IL-6 and osteocalcin genes are located on chromosomes 7p21 and 1q25-q31, respectively, and no significant relation between the distributions of the corresponding genotypes was detected. Given the small number of subjects with the GG/CC genotype (n = 4), it was excluded from the analysis of combined genotype. Age and BMI did not differ among the remaining eight combined genotypes in premenopausal (data not shown) or postmenopausal (Table 9) women. For premenopausal women, there were no differences in BMD at the sites examined among combined genotypes (data not shown). For postmenopausal women, BMD for the total body, lumbar spine, or Ward's

TABLE 6. BMD and other characteristics of men (n = 1130) according to VDR genotype

Characteristic	CC	CT	TT
Number (%)	448 (39.7)	520 (46.0)	162 (14.3)
Age (yr)	58.7 ± 0.5	59.7 ± 0.5	59.2 ± 0.9
BMI (kg/m^2)	22.9 ± 0.1	22.9 ± 0.1	23.0 ± 0.2
BMD values measured by pQCT (mg/cm^3)			
D50	273.2 ± 3.1	263.5 ± 2.9[a]	261.1 ± 5.1
D100	546.4 ± 4.4	537.2 ± 4.1	537.9 ± 7.2
P100	1190.9 ± 6.7	1181.7 ± 6.2	1183.1 ± 11.0
BMD values measured by DXA (g/cm^2)			
Total body	1.094 ± 0.004	1.082 ± 0.004	1.089 ± 0.007
L2–L4	0.988 ± 0.007	0.979 ± 0.007	0.985 ± 0.012
Femoral neck	0.763 ± 0.005	0.747 ± 0.004[a]	0.748 ± 0.008
Trochanter	0.677 ± 0.005	0.663 ± 0.004	0.664 ± 0.008
Ward's triangle	0.563 ± 0.006	0.544 ± 0.005[a]	0.548 ± 0.009

Data are expressed as means ± SE. BMD values were adjusted for age and BMI.
[a] $P \leq 0.05$ *vs.* CC.

triangle was lower in those with the GG/*TT* genotype than in those with the CC/CC, CC/CT, CC/*TT*, CG/CC, CG/CT, or CG/*TT* genotypes. The differences in BMD for the total body between the GG/*TT* and CC/CC genotypes, for the lumbar spine between GG/*TT* and CG/CC genotypes, and for Ward's triangle between GG/*TT* and CC/CC genotypes in postmenopausal women were 8.6, 17.7, and 24.0%, respectively.

Discussion

Given that selection bias can influence the results of association studies, it is important that study populations be genetically and ethnically homogeneous. Our study population was recruited randomly from individuals resident in Obu city and Higashiura town in central Japan, where the population is thought to share the same ethnic ancestry and to possess a homogeneous genetic background (20). We also showed that the genotype distributions of the IL-6, osteocalcin, and VDR genes were in Hardy-Weinberg equilibrium both for women and for men. Our study population therefore appeared genetically homogeneous, and we thus appeared to avoid admixture and selection bias.

For the −634C→G SNP of the IL-6 gene, BMD was reduced in postmenopausal women with the GG genotype compared with that in those with the CC or CG genotypes, consistent with the results of a previous study of 470 postmenopausal Japanese women (9). In this previous study, however, only radial BMD was measured (9). Our results now show that IL-6 genotype is associated with BMD not only of the distal radius but also of the total body, lumbar spine, femoral neck, trochanter, and Ward's triangle in a large population of postmenopausal Japanese women.

For the 298C→T SNP of the osteocalcin gene, BMD decreased according to the rank order of genotypes CC > *CT* > *TT* in postmenopausal women. This observation differs from previous results obtained with 160 postmenopausal Japanese women showing that BMD of the lumbar spine increased according to the rank order of genotypes CC < *CT* < *TT*, although the observed differences were not statistically significant (12). Our present results demonstrate an association of osteocalcin genotype with BMD for the total body, femoral neck, trochanter, and Ward's triangle as well as for the lum-

TABLE 7. Effects of IL-6, osteocalcin, and VDR genotypes as well as of other characteristics on BMD for women and men determined by multivariate regression analysis

	Women						Men				
BMD value	Age (yr)	BMI (kg/m^2)	Menstrual state	IL-6 genotype	Osteocalcin genotype	VDR genotype	Age (yr)	BMI (kg/m^2)	IL-6 genotype	Osteocalcin genotype	VDR genotype
D50	0.325 (<0.001)	0.017 (<0.001)	0.010 (<0.001)	NS	0.004 (0.009)	NS	0.105 (<0.001)	0.019 (<0.001)	NS	NS	0.005 (0.014)
D100	0.492 (<0.001)	0.007 (<0.001)	0.011 (<0.001)	NS	NS	NS	0.155 (<0.001)	0.007 (0.003)	NS	NS	NS
P100	0.549 (<0.001)	0.003 (0.005)	0.005 (0.001)	NS	NS	NS	0.166 (<0.001)	NS	NS	NS	NS
Total body	0.555 (<0.001)	0.010 (<0.001)	0.011 (<0.001)	0.003 (0.011)	0.004 (0.001)	NS	0.050 (<0.001)	0.075 (<0.001)	NS	0.004 (0.027)	NS
L2–L4	0.396 (<0.001)	0.057 (<0.001)	0.022 (<0.001)	0.004 (0.007)	0.003 (0.010)	NS	NS	0.137 (<0.001)	NS	NS	NS
Femoral neck	0.405 (<0.001)	0.063 (<0.001)	0.002 (0.046)	NS	0.002 (0.043)	NS	0.083 (<0.001)	0.157 (<0.001)	NS	NS	0.004 (0.018)
Trochanter	0.386 (<0.001)	0.082 (<0.001)	NS	NS	0.002 (0.035)	NS	0.016 (<0.001)	0.176 (<0.001)	NS	NS	0.003 (0.051)
Ward's triangle	0.498 (<0.001)	0.031 (<0.001)	0.003 (0.006)	NS	0.004 (0.004)	NS	0.220 (<0.001)	0.060 (<0.001)	NS	NS	0.003 (0.041)

Data are R^2 (*P*) values from multivariate regression analysis of age, BMI, menstrual state (0 = premenopause; 1 = postmenopause), IL-6 genotype (0 = CC; 1 = CG; 2 = GG), osteocalcin genotype (0 = CC; 1 = CT; 2 = TT), and VDR genotype (0 = CC; 1 = CT; 2 = TT). NS, Not significant.

TABLE 8. Distribution of IL-6 and osteocalcin genotypes among women

Osteocalcin genotype	IL-6 genotype			Total
	CC	CG	GG	
CC	28 (2.58%)	18 (1.66%)	4 (0.37%)	50 (4.60%)
CT	196 (18.03%)	135 (12.42%)	19 (1.75%)	350 (32.20%)
TT	393 (36.15%)	248 (22.82%)	46 (4.23%)	687 (63.20%)
Total	617 (56.76%)	401 (36.89%)	69 (6.35%)	1087 (100%)

bar spine in a large female population, with the CC genotype exhibiting the highest BMD and the *TT* genotype the lowest BMD.

The 2C→T SNP at the translation initiation site of the VDR gene has previously been shown to be associated with BMD in postmenopausal Japanese women, with the *TT* genotype implicated as a risk factor for reduced BMD (15). However, we did not detect an association between this SNP and BMD for community-dwelling women. This SNP was also previously associated with BMD in small populations of Caucasian men, with the *T* allele being a predisposing factor to reduced bone mass (17, 18). Our results demonstrate that BMD for the distal radius (D50), femoral neck, or Ward's triangle was significantly reduced in men with the *CT* genotype compared with that in those with the CC genotype.

Analysis of combined IL-6 and osteocalcin genotypes revealed that the largest differences in BMD for the total body, lumbar spine, and Ward's triangle in postmenopausal women were 8.6, 17.7, and 24.0%, respectively, indicating that the effects of the two SNPs on BMD are additive. These results thus suggest that combined genotypes may be more useful for predicting bone mass in postmenopausal women.

The molecular mechanisms that underlie the association of the −634C→G SNP of the IL-6 gene and the 298C→T SNP of the osteocalcin gene with BMD in postmenopausal women remain unclear. The effects of these SNPs on the transcriptional activity of the corresponding gene promoters have not been determined. The 2C→T SNP of the VDR gene was shown to affect the molecular mass of the encoded protein (*T* allele, 50 kDa; C allele, 49.5 kDa) as well as the transcriptional activation of the gene by vitamin D (*T* allele < C allele) (15). These observations, however, were not independently confirmed (21). The functional impact of this polymorphism of the VDR gene thus remains to be determined.

Given the multiple comparisons of genotypes with BMD in the present study, it is not possible to completely exclude potential statistical errors such as false positives. It is also possible that the SNPs examined in our study are in linkage disequilibrium with polymorphisms of other nearby genes that are located at chromosome 7p21 (IL-6 gene), 1q25-q31 (osteocalcin gene), or 12q12-q14 (VDR gene) and that they are actually responsible for the association with BMD. However, our present results suggest that the IL-6 and osteocalcin genes are susceptibility loci for reduced bone mass in postmenopausal Japanese women and that the VDR gene constitutes such a locus in Japanese men. The combined IL-6 and osteocalcin genotypes exhibited an additive effect on BMD and may thus prove informative for the assessment of osteoporosis in women.

TABLE 9. BMD and other characteristics of postmenopausal women (n = 807) according to combined IL-6 and osteocalcin genotypes

Characteristic	Genotype (IL-6/osteocalcin)							
	CC/CC	CC/CT	CC/TT	CG/CC	CG/CT	CG/TT	GG/CT	GG/TT
Number (%)	20 (2.48)	143 (17.72)	287 (35.56)	14 (1.73)	111 (13.75)	185 (22.92)	10 (1.24)	37 (4.58)
Age (yr)	61.8 ± 1.9	63.1 ± 0.7	64.6 ± 0.5	59.6 ± 2.3	63.6 ± 0.8	63.6 ± 0.6	63.0 ± 2.7	66.4 ± 1.4
BMI (kg/m^2)	22.6 ± 0.7	23.1 ± 0.3	23.0 ± 0.2	24.8 ± 0.9	22.6 ± 0.3	22.8 ± 0.2	21.9 ± 1.1	23.5 ± 0.5
BMD values measured by pQCT (mg/cm^3)								
D50	187.0 ± 13.9	169.1 ± 5.3	162.2 ± 3.8	174.3 ± 16.7	170.4 ± 6.0	160.5 ± 4.7	137.3 ± 19.7	138.7 ± 10.7
D100	473.0 ± 20.1	445.3 ± 7.6	443.0 ± 5.4	440.0 ± 24.1	452.4 ± 8.7	442.9 ± 6.8	431.6 ± 28.4	404.5 ± 15.4
P100	1129.0 ± 33.2	1074.8 ± 12.6	1084.6 ± 8.9	1081.4 ± 39.8	1092.0 ± 14.3	1073.7 ± 11.2	1070.5 ± 46.9	1029.4 ± 25.5
BMD values measured by DXA (g/cm^2)								
Total body	0.954 ± 0.019[a]	0.933 ± 0.007[a]	0.917 ± 0.005[b]	0.946 ± 0.022	0.916 ± 0.008	0.915 ± 0.006	0.882 ± 0.028	0.872 ± 0.014
L2–L4	0.864 ± 0.028[a]	0.820 ± 0.011[a]	0.807 ± 0.007[b]	0.891 ± 0.034[a]	0.813 ± 0.012[b]	0.808 ± 0.009[b]	0.737 ± 0.042	0.733 ± 0.021
Femoral neck	0.674 ± 0.019	0.653 ± 0.007	0.641 ± 0.005	0.682 ± 0.022	0.644 ± 0.008	0.645 ± 0.006	0.609 ± 0.028	0.607 ± 0.014
Trochanter	0.576 ± 0.018	0.551 ± 0.007	0.537 ± 0.005	0.569 ± 0.022	0.539 ± 0.008	0.538 ± 0.006	0.484 ± 0.027	0.515 ± 0.013
Ward's triangle	0.530 ± 0.026[a,c]	0.461 ± 0.010	0.450 ± 0.007	0.516 ± 0.031[b]	0.449 ± 0.011	0.452 ± 0.008	0.427 ± 0.038	0.403 ± 0.019

Data are expressed as means ± SE. BMD values were adjusted for age and BMI.
[a] $P \leq 0.005$ *vs.* GG/TT; [b] $P \leq 0.05$ *vs.* GG/TT; [c] $P \leq 0.05$ *vs.* CC/TT.

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PAPER

Association of polymorphisms in the estrogen receptor α gene with body fat distribution

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OBJECTIVE: To examine whether polymorphisms of the estrogen receptor (ER) α gene are associated with body fat distribution.
DESIGN: Cross-sectional, epidemiological study of two single-nucleotide polymorphisms, a T→C (*PvuII*) and an A→G (*XbaI*), in the first intron of the ERα gene.
SUBJECTS: A total of 2238 community-dwelling middle-aged and elderly Japanese population (age: 40–79 y).
MEASUREMENTS: The ERα genotypes (by automated fluorescent allele-specific DNA primer assay system), anthropometric variables, fat mass (FM) and percentage FM (%FM) (by dual-energy X-ray absorptiometry).
RESULTS: FM and waist were inversely associated with age ($r=-0.630$ and -0.504, respectively) in women with the *GG* genotype. On the other hand, waist circumference of the *AA* genotype was positively correlated with age ($r=0.231$). Thus, for middle-aged women (40–59 y) with the *AG* or *GG* genotype body mass index (BMI), %FM, FM, waist, hip and waist-to-hip ratio (WHR) were larger than those with the *AA* genotype. In particular, FM and waist were greater by 20% and 9%, respectively, for the *GG* genotype, compared to the *AA* genotype. Alternatively, FM and waist were smaller by 18% and 6%, respectively, in older women with the *GG* genotype, compared to the *AA* genotype. No effect was found among the A→G polymorphisms for men. For both genders, no difference was found in any variables among the *TT*, *TC* and *CC* genotypes with the exception of BMI of older men (60–79 y).
CONCLUSION: No association was found between the ERα gene polymorphisms and body fat distribution in men. For women, the A→G polymorphism, in particular the *GG* genotype, may contribute to the development of upper-body obesity in middle-aged individuals, but may serve to decrease the whole-body and abdominal fat tissue of older individuals.
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Keywords: estrogen receptor gene; polymorphism; body composition; waist; waist-to-hip ratio

Introduction

It has been found that body fat distribution is an important factor in coronary heart disease (CHD). In particular, a large waist circumference or waist-to-hip ratio (WHR) is closely associated with an increased prevalence of risk factors for CHD, for example, impaired glucose tolerance, insulin resistance, lipoprotein metabolic disorder and hypertension.[1–3] Though, in general, upper-body or android-type[4] obesity, with a large waist or WHR, is more frequently observed in men compared with women, this obesity phenotype is also observed fairly often in postmenopausal women.[5] This is because estrogen deficiency during the normal menopausal transition accelerates the selective deposition of intra-abdominal fat.[6] With respect to estrogen's association to body fat distribution, several authors [6–10] have reported that estrogen hormone replacement therapy had desirable effects on body fat distribution in postmenopausal women. These findings suggest that estrogen plays an important role in the modification of body fat distribution.

More recently, associations have been found between estrogen receptor (ER) α gene polymorphism and bone mineral density,[11–18] pathogenesis of type II diabetes,[19] and susceptibility to or age of onset of autoimmune diseases such as multiple sclerosis.[20] The human ERα gene is located on chromosome 6p25.1, is comprised of eight exons, and spans

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>140 kb.[21] Two single-nucleotide polymorphisms (SNPs) have been identified in the first intron of the ERα gene: a T→C polymorphism that is recognized by the restriction endonuclease *Pvu*II (*T* and *C* alleles correspond to the presence (*p* allele) and absence (*P* allele) of the restriction site, respectively) and an A→G polymorphism that is recognized by *Xba*I (*A* and *G* alleles correspond to the presence (*x* allele) and absence (*X* allele) of the restriction site, respectively). According to studies on the relation between the ER gene polymorphism and bone mineral density, the SNPs, alone or in combination, were associated with lower bone mineral density in pre- and postmenopausal women,[11,14,15,17,18,22,23] and were important factors in determining changes in bone mass in older women receiving hormone replacement therapy.[16,24]

To our knowledge, little has been reported on the association between the ER gene polymorphisms and body fat distribution. In clinical settings for obesity treatment, understanding this association would be helpful not only for early preventative treatment of upper-body obesity but also for predicting the effects of estrogen replacement therapy on the modification of body fat distribution. The purpose of this study, therefore, was to examine whether the T→C (*Pvu*II) and A→G (*Xba*I) polymorphisms of the ERα gene, alone or in combination, are associated with body fat distribution in a middle-aged to elderly Japanese population.

Methods

Subjects

There were 1110 women and 1128 men who participated in the first wave of examinations in the National Institute for Longevity Sciences–Longitudinal Study of Aging (NILS-LSA) from April 1998 to March 2000. There were randomly sampled, community-dwelling individuals aged 40–79 y, stratified by age and gender and living in the neighborhood of the NILS. Details of the NILS-LSA have been described elsewhere.[25] The aim and design of the study were explained to each subject before they gave their written informed consent. The study was approved by the Committee of the Chubu National Hospital.

Determination of ERα genotypes

The ERα genotypes were determined in accordance with a study by Yamada *et al*.[26]. The ERα gene was analyzed with an automated fluorescent allele-specific DNA primer assay system (Toyobo Gene Analysis, Osaka, Japan). To determine the T→C (*Pvu*II) genotype, the polymorphic region of the gene was amplified by polymerase chain reaction with allele-specific sense primers labeled at the 5′ end either with fluorescein isothiocyanate (5′-AGTTCCAAATGTCC-CAGXTG-3′) or with Texas red (5′-AGTTCCAAATGTCC-CAGXCG-3′) and an antisense primer labeled at the 5′ end with biotin (5′-TCTGGGAAACAGAGACAAAGC-3′). 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 mmol/l $MgCl_2$, and 1 U of DNA polymerase (rTaq; Toyobo) in rTaq buffer. The amplification protocol consisted of three parts: initial denaturation at 95°C for 5 min; 35 cycles of denaturation at 95°C for 30 sec, annealing at 62.5°C for 30 sec, and extension at 72°C for 30 sec; and a final extension at 72°C for 2 min.

To determine the A→G (*Xba*I) genotype, the polymorphic region of the gene was amplified by polymerase chain reaction with a sense primer labeled at the 5′ end with biotin (5′-CTGTTTCCCAGAGACCCTGAG-3′) and allele-specific antisense primers labeled at the 5′ end either with fluorescein isothiocyanate (5′-CCAATGCTCATCCCAACTX-TA-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 the T→C (*Pvu*II) 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 supernatants were then collected from each well, transferred to the wells of a 96-well plate containing 0.01 M NaOH, and 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 at 584 and 612 nm, respectively, for Texas red.

The T→C and A→G polymorphisms were determined in 2228 subjects (1108 women, 1120 men) and in 2235 subjects (1107 women, 1128 men), respectively.

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 body mass index (BMI) was calculated as weight (kg) divided by height squared (m^2). Waist circumference and WHR were used as the indices for body fat distribution in this study. WHR was calculated as a ratio of waist circumference measured at the level of the umbilicus to hip circumference.

Body composition by dual-energy X-ray absorptiometry

Whole-body fat mass (FM), fat-free mass (FFM) and percentage FM (%FM), assessed by dual-energy X-ray absorptiometry (QDR-4500; Hologic, Madison, OH, USA), were used as the indices for determining body composition. Transverse scans were used to measure FM and FFM, and pixels of soft tissue were used to calculate the ratio (*R* value) of mass attenuation coefficients at 40–50 keV (low energy) and 80–100 keV (high energy), using software version 1.3Z.

Physical activity, smoking status, menstrual status and hormonal replacement therapy

A detailed interview with questionnaire sheets [27,28] revealed work time and leisure time physical activities of the subjects. Amount of physical activity was calculated as a product of the metabolic-equivalent (MET) by duration in minutes. Smoking status, menstrual status and hormonal replacement therapy were examined by a medical doctor. Menopause was defined as the absence of menses for at least 12 months by a questionnaire.

Biochemical assays of blood

An antecubital blood sample was drawn from each subject after an overnight fast. Serum total cholesterol and triglycerides were determined enzymatically, serum high-density lipoprotein cholesterol was measured by the heparin-manganese precipitation method and fasting plasma glucose was assayed by a glucose oxidase method. Plasma insulin was measured in duplicate by radioimmunoassay. Serum low-density lipoprotein cholesterol was estimated according to the Friedewald formula.[29]

Data analysis

To examine whether age may influence the relation between the ERα gene polymorphisms and body fat distribution, we subdivided each gender group into two age groups: middle-aged (40–59 y) and older (60–79 y). Furthermore, middle-aged women were categorized by menopause status. Values are expressed as mean ± standard error (s.e.) in the tables and figures. Allele frequencies were estimated by the gene-counting method, and the χ^2 test was used to identify significant departures from Hardy–Weinberg equilibrium. The distribution of haplotypes for the T→C and A→G polymorphisms was calculated according to the method by Thompson *et al*.[30] The data were compared by one-way analysis of variance and the Tukey–Kramer *post hoc* test. When a significant difference exists, analysis of covariance was used with age, smoking status, menstrual status, hormonal replacement therapy and physical activities as covariates. The relations between age and both FM and waist were tested by correlation analysis. In each statistical analysis, probability values below 0.05 were regarded as significant. The data were analyzed with the Statistical Analysis System (SAS), release 6.12.

Results

Physical and biochemical blood characteristics of the subjects are shown for each gender in Table 1. No difference was found in BMI, waist, hip or WHR between genders, whereas %FM and FM were significantly greater in women than in men. The distributions of ERα genotypes with regard to the T→C and A→G SNPs were in Hardy–Weinberg equilibrium for the subjects (see Table 2). There was no difference in the distribution between genders or between age groups. The distribution of haplotypes for the T→C and A→G polymorphisms in all study subjects was as follows: *T/A*, 61.9%; *T/G*, 0.2%; *C/A*, 28.6%; and *C/G*, 9.3%. The T→C and A→G SNPs were in linkage disequilibrium (pairwise linkage disequilibrium coefficient, D' (D/D_{max}), of 0.97; standardized linkage disequilibrium coefficient, r, of 0.40; $P<0.0001$, χ^2 test).

Table 1 Physical and biochemical blood characteristics of subjects ($n=2238$)

	Women	*Men*
Number	1110	1128
Age(y)	59.3±0.3	59.2±0.3
Body mass index(Kg/m^2)	22.9±0.1	22.9±0.1
Percent fat mass(%)	31.5±0.2	21.3±0.1[a]
Fat mass(Kg)	16.8±0.1	13.5±0.1[a]
Fat-free mass(Kg)	35.7±0.1	48.7±0.2[b]
Waist(cm)	83.7±0.3	84.2±0.3
Hip(cm)	90.7±0.2	91.1±0.2
Waist-to-hip ratio	0.92±0.002	0.92±0.002
Total cholestrol(mg/dl)	226.9±1.1	212.2±1.0[a]
Triglycerides(mg/dl)	109.2±1.8	133.9±2.9[b]
High-density lipoprotein cholestrol (mg/dl)	66.0±0.5	57.4±0.4[a]
Low-density lipoprotein cholestrol (mg/dl)	139.4±1.1	131.8±1.0[a]
Glucose(mg/dl)	100.7±0.6	105.8±0.7[b]
Insulin (μ/ml)	8.4±0.2	8.3±0.2

Data are expressed as mean ± s.e.
[a]significantly larger or higher in women than in men.
[b]significantly smaller or lower in women than in men.

Table 2 Distribution of T→C(*Pvu* II) and A→G(*Xba* I) genotypes of the ERα gene of the subjects

	AA	*AG*	*GG*	*Total*
TT	796	1	0	787
	35.7%	0.04%	0.0%	35.8%
TC	587	466	5	1058
	26.4%	20.9%	0.2%	47.5%
CC	123	175	73	371
	5.5%	7.9%	3.3%	16.7%
Total	1506	642	78	2226
	67.6%	28.9%	3.5%	100%

T→C polymorphisms (*PvuII*)

For both genders, no significant difference was found in any variables among the T→C polymorphisms with the exception of BMI of the older men (table not shown). Means ± s.e. of BMI of the older men were 22.9±0.2, 22.5±0.2 and 22.0±0.3 kg/m^2 in the *TT*, *TC* and *CC* genotypes, respectively (*TT*>*CC*, $P=0.025$).

Table 3 Physical status and Physical activities of women according to age group and A→G genotype ($n=1107$)

	Middle-aged ($n=551$)			Older($n=556$)		
	AA	*AG*	*GG*	*AA*	*AG*	*GG*
Number(%)	376(68.2%)	156(28.3%)	19(3.5%)	362(65.1%)	174(31.3%)	20(3.6%)
Age(y)	49.7±0.3	49.8±0.4	50.1±1.2	68.5±0.3	69.1±0.4	69.9±1.2
Body mass index(Kg/m^2)	22.4±0.2	23.4±0.3	24.4±0.7[a]	23.0±0.2	23.3±0.3	21.8±0.7
Percent fat mass(%)	30.1±0.2	31.3±0.4	33.6±1.1[b]	32.4±0.3	33.0±0.4	29.7±1.2[g]
Fat mass(Kg)	16.2±0.2	17.7±0.4	19.5±1.1[c]	16.8±0.3	17.1±0.4	14.0±1.1[h]
Fat-free mass(Kg)	37.0±0.2	37.8±0.3	38.0±0.9	34.2±0.2	33.8±0.3	32.3±0.9
Waist(cm)	81.1±0.5	83.6±0.7	88.0±2.0[d]	85.5±0.5	85.7±0.8	80.6±2.2
Hip(cm)	91.0±0.3	92.5±0.4	93.2±1.2[e]	89.9±0.3	90.0±0.4	87.2±1.3
Waist-to-hip-ratio	0.89±0.003	0.90±0.005	0.94±0.015[f]	0.95±0.004	0.95±0.006	0.92±0.016
Leisure time physical activity (mets*min/day)	27113±1931	27837±2952	15505±8720	37297±2541	30823±3671	31128±10765
Work time physical activity (mets*min/day)	326405±6660	320074±10307	330451±29627	215244±6320	220031±9117	204837±26814

Data are expressed as means±S.E.
[a]*AG>AA* ($P=0.0015$), *GG>AA* ($P=0.0181$).
[b]*AG>AA* ($P=0.0316$), *GG>AA* ($P=0.0061$).
[c]*AG>AA* ($P=0.0023$), *GG>AA* ($P=0.0089$).
[d]*AG>AA*($P=0.0059$), *GG>AA* ($P=0.0024$).
[e]*AG>AA* ($P=0.0084$).
[f]*AG>AA* ($P=0.0018$), *GG>AG* ($P=0.0316$).
[g]*AG>GG* ($P=0.0232$).
[h]*AG>AA*($P=0.0323$), *AG>GG* ($P=0.0221$).

A→G polymorphism (*XbaI*)

For middle-aged women with the *AG* and/or *GG* genotypes, BMI, %FM, FM, waist, hip and WHR were greater than in the middle-aged women with the *AA* genotype (Table 3). In particular, those with the *GG* genotype had a 9% greater BMI, a 20% greater FM and a 9% larger waist compared with the *AA* genotype. Fasting insulin was significantly higher in the individuals with the *AG* genotype (8.3±0.3 μU/ml), compared with the *AA* genotype (7.4±0.2 μU/ml) (see Figure 1). No difference was found in plasma lipids and fasting blood glucose among the genotypes. When the analysis of covariance with age, smoking status, menstrual status, hormonal replacement therapy and physical activities as covariates was used, these results remained essentially unchanged with the exception of hip circumference and WHR ($P<0.1$). Significant differences ($P<0.05$) were still observed in BMI (*AA* 22.3±0.3, *AG* 23.7±0.4, *GG* 24.4±1.0: *AG* and *GG* > *AA*), %FM (*AA* 29.9±0.4, *AG* 31.9±0.6, *GG* 33.1±1.5: *AG* and *GG* > *AA*), FM (*AA* 16.0±0.4, *AG* 18.2±0.6, *GG* 19.1±1.5: *AG* and *GG* > *AA*), waist (*AA* 81.3±0.7, *AG* 85.5±1.1, *GG* 88.2±2.5: *AG* and *GG* > *AA*) and fasting insulin (*AA* 7.3±0.2, *AG* 8.4±0.4, *GG* 8.0±0.9: *AG* > *AA*) among the genotypes. For older women with the *GG* genotype, %FM, FM and waist were smaller by 10, 18 and 6%, respectively, compared to the older women with the *AA* and/or *AG* genotypes. These results were also unchanged when the analysis of covariance was used. Significant differences ($P<0.05$) were still observed in %FM (*AA* 32.1±0.5, *AG* 33.7±0.7, *GG* 27.5±1.8: *AA* and *AG* > *GG*) and FM (*AA* 16.4±0.4, *AG* 18.1±0.6, *GG* 13.4±1.6: *AA* and *AG* > *GG*) among the genotypes. No difference was found in any variables among the genotypes in men (table was not shown).

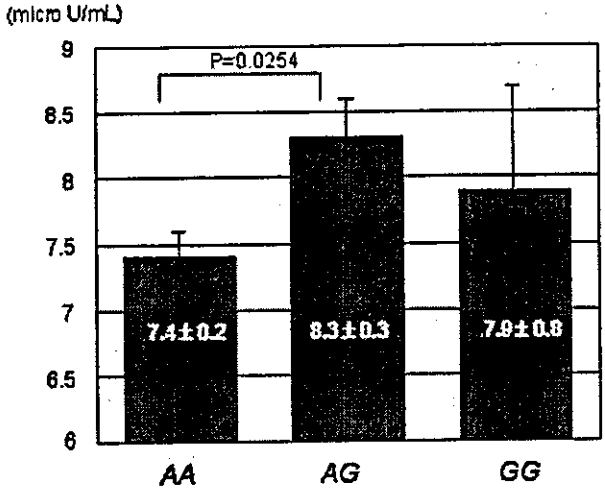

Figure 1 Comparison of fasting insulin levels among A→G genotypes in middle-aged women.

Table 4 compares age, FM, waist and WHR among the A→G genotypes in middle-aged women according to menstrual status. Women who could not be clearly defined as pre- or postmenopausal were excluded from this analysis. For premenopausal women with the *GG* genotype, FM, waist and WHR were larger than in those with the *AA* genotype,

Table 4 Comparison of age, fat mass and waist among the A→G genotypes in middle-aged women according to menstrual status ($n=536$)

	Premenopausal ($n=277$)			*Postmenopausal ($n=259$)*		
	AA	*AG*	*GG*	*AA*	*AG*	*GG*
Number(%)	192(69.3%)	74(26.7%)	11(4.0%)	169(65.4%)	82(31.5%)	8(3.1%)
Age(y)	46.0±0.2	45.5±0.4	46.3±1.3	53.8±0.3	53.6±0.4	55.4±0.9
Fat mass(Kg)	15.9±0.3	17.4±0.6	20.9±1.5[a]	16.6±0.3	18.1±0.6	17.5±0.9
Waist(cm)	80.0±0.6	82.8±1.1	89.3±2.9[b]	82.2±0.5	84.4±1.1	86.3±2.8
Waist-to-hip ratio	0.87±0.004	0.89±0.008	0.94±0.021[c]	0.91±0.005	0.91±0.007	0.94±0.015

Data are expressed as means±s.e.
[a]*GG>AA* ($P=0.0085$).
[b]*GG>AA* ($P=0.0021$).
[c]*GG>AA* ($P=0.0025$),GG>AG ($P=0.0406$).

whereas no difference was found in postmenopausal women. These results also remained essentially unchanged with the exception of WHR ($P<0.1$) when the analysis of covariance with age and smoking status as covariates was used. Significant differences ($P<0.05$) were still observed in FM (*AA* 15.8±0.5, *AG* 18.0±0.7, *GG* 20.7±1.6: *GG* > *AA*) and waist (*AA* 80.2±0.7, *AG* 83.7±1.3, *GG* 88.5±3.2: *GG* > *AA*) among the genotypes.

Combination of the T→C and A→G polymorphisms

To determine whether the T→C and A→G polymorphisms synergistically influence body fat distribution, we compared the variables by combined genotypes (table not shown). Because of the small number of subjects, *TT/AG* ($n=1$), *TT/GG* ($n=0$) and *TC/GG* ($n=5$) were excluded from this analysis.

For middle-aged women with the *CC/GG* genotype, the mean values of BMI, FM and waist were significantly larger by 8%, 18% and 8%, respectively, compared to those with the *TT/AA* genotype. But the differences between the *CC/GG* and *TT/AA* genotypes were similar to the differences between the A→G polymorphism (see Table 3). The physical activities did not differ between the *CC/GG* and *TT/AA* genotypes. For older women with the *CC/GG* genotype, the mean values of %FM and FM were significantly lower by 12% and 17%, respectively, compared to those with the *TT/AA* genotype. These results also indicate that the effects of the combined genotypes on %FM and FM are not different from the A→G polymorphism alone. For both middle-aged and older men, no difference was found in any variables among the combined genotypes.

Relation of age with FM and waist

Figure 2 shows the relations between age and both FM and waist in combined data from middle-aged and older women. A significant and inverse correlation ($r=-0.630$, $P<0.001$) was found between FM and age in the group with the *GG* genotype. Waist circumference was positively associated with age ($r=0.231$, $P<0.001$) in the group with the *AA* genotype, whereas an inverse association ($r=-0.504$, $P<0.001$) was found in the group with the *GG* genotype.

Discussion

Although we have reported in the previous study [26] that the A→G polymorphism of the ERα gene may be associated with a greater BMI in middle-aged women, little is known about the association between the ERα gene polymorphisms and body fat distribution or body composition. Rankinen *et al*[31] reported in the eighth update of the human obesity gene map that 174 studies found positive associations of obesity phenotype with 58 candidate genes, but no more than one study by Speer *et al*[19] showed any association between ERα gene polymorphisms and obesity phenotype. According to the study, 29 subjects (23 women and 6 men) with android-type obesity, 69 and 31% had the *AG* and *GG* genotypes, respectively, and the *AA* genotype was not found.

One of the major findings of the present study is to find the association of the A→G polymorphism or the combination of the T→C and A→G polymorphisms with not only a greater BMI but also larger %FM, FM, waist circumference and WHR in middle-aged women. Moreover, the results of Table 4 reveal that for premenopausal women, the effect of the ERα gene polymorphisms on body fat distribution (FM and waist) was more significant than for postmenopausal women. These observations suggest that the greater FM of individuals with the gene mutation were due to the selective fat accumulation at the abdomen, especially the intra-abdominal cavity. Further studies on the association between the gene polymorphisms and amount of intra-abdominal fat are needed to clarify the above speculation.

An increasing fasting insulin is induced by an excess accumulation of abdominal fat.[32] In addition, Cooke *et al*[33] found that knocked-out ERα caused adipocyte hyperplasia and hypertrophy in white adipose tissue, and is accompanied by insulin resistance and glucose intolerance in rats.[33] On the basis of the above studies, we expected that fasting

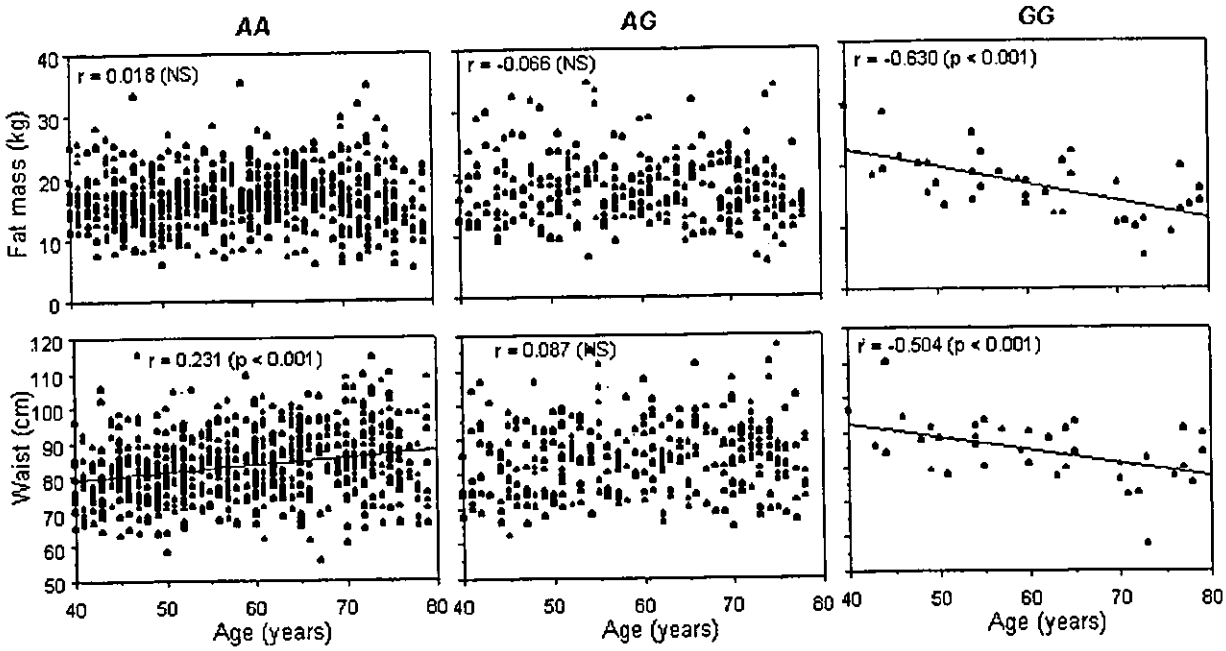

Figure 2 Relations of age with fat mass and waist by each *A→G* genotype in women.

insulin level in the *GG* genotype would be highest of all the genotypes. However, as illustrated in Figure 1, fasting insulin level in the *GG* genotype did not always indicate the highest values. Recently, Snijder *et al* [34] reported that fasting insulin level was positively correlated with waist circumference but was inversely associated with hip circumference. Subjects with the *GG* genotype have not only larger waist but also larger hip compared with the *AG* genotype. Thus, their fasting insulin levels might be influenced by the two opposite functions with each other.

Regardless of the strong association between the A→G polymorphism and BMI, FM, waist or WHR in middle-aged women (see Table 3), the T→C polymorphism alone was not associated with any variables. Moreover, additive and synergistic effects of the genotypes were not apparent with regard to body fat distribution or body composition in the subjects. These results suggest that the A→G polymorphism plays an important role in body fat distribution and body composition in middle-aged women, but the T→C polymorphism does not.

Figure 2 shows that the waist circumference of women with the *AA* genotype increased with age, which is expected. This indicates a natural (normal) change in body fat distribution of middle-aged and older women.[5] In contrast, results of the *GG* genotype revealed that FM and waist size were inversely associated with age. Consequently, our data suggest that (1) middle-aged women with the *GG* genotype presented with a larger FM and waist, and (2) older women with the *GG* genotype presented with a smaller FM and waist, compared with the *AA* genotype, despite the observation in the middle-aged women (see Table 3). Estrogen plays an important role in maintaining desirable fat distribution in premenopausal women. Therefore, when a functional change of the ERα was induced by the gene mutation, the estrogen sensitivity is deteriorated, which possibly caused the android-type fat distribution in middle-aged and premenopausal women with the *GG* genotype (see Table 4). On the other hand, the smaller FM and waist of older women with the *GG* genotype have been possibly induced by some specific effects of the gene mutation; however, the mechanisms cannot be explained by the data from this study.

Both the T→C and A→G polymorphisms are found in intronic regions. Intronic changes in gene sequence may have an impact on the expression of other genes by influencing the transcription and/or stability of mRNA of those genes.[35,36] Thus, further studies on the relations of ERα gene polymorphisms and body fat distribution are needed to validate the findings of this study.

Our data raise the possibility that the A→G polymorphisms of the ERα gene, especially the *GG* genotype, contribute to development of the android-type fat distribution in middle-aged and premenopausal women. In older women, this gene polymorphism may serve to decrease whole-body and abdominal fat tissue.

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ORIGINAL INVESTIGATION

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Association of the mitochondrial DNA 15497G/A polymorphism with obesity in a middle-aged and elderly Japanese population

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Abstract Although polymorphism of the mitochondrial DNA 15497guanine/adenine (Mt15497G→A) leads to the Gly251Ser amino acid replacement on human cytochrome *b*, it is unknown whether functional alteration of the mitochondrion is induced by the Gly251Ser replacement. To see if an association exists between the Mt15497G→A polymorphism and obesity, we examined differences in body size, body composition, and regional body fat distribution between the two genotypes in middle-aged and elderly Japanese individuals (825 women and 906 men). The Mt15497 genotype was determined with an automated colorimetric allele-specific DNA probe assay system using the polymerase chain reaction (PCR) method. The Mt15497G→A polymorphism was detected in 3.5% (n=60) of all subjects: 2.8% (n=23) among women and 4.1% (n=37) among men. After adjusting for age and smoking, we found that body weight, body mass index, waist and hip circumferences, fat mass, fat-free mass, intra-abdominal fat and triglycerides were significantly greater in women with the A allele compared with the G allele (p=0.001–0.025). For men, waist to hip ratio was significantly greater (p=0.032), and waist circumference, intra-abdominal fat and triglycerides had a trend to be significantly greater (p=0.062–0.087) in subjects with the A allele compared with the G allele. These data suggest that the Mt15497 polymorphism may be associated with obesity-related variables and lipid metabolism.

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Introduction

Obesity probably develops through interaction of both genetic and environmental factors. Polymorphisms of leptin, the UCP family, and beta 3 adrenergic receptor genes are examples of some of the genetic factors predisposing individuals to obesity (Bouchard et al. 1988). Variations in mitochondrial (mt) DNA also have emerged as possible genetic factors that lead to a high BMI (Rowe et al. 1991). Hegel et al. (1997) and Kokaze et al. (2001) reported on the association between plasma triglycerides concentration and polymorphisms of mtDNA 16517 and 5178, respectively. They speculated that a polymorphism of mtDNA may partially alter the function of the mitochondrial β-oxidation of fatty acid. Moreover, qualitative change in the mitochondria may affect the function of the TCA cycle and energy consumption within skeletal muscle. Hence, functional alterations of mtDNA could facilitate development of obesity. Dionne et al. (1992) observed in a monozygotic twin study that the mtDNA D-loop *Kpn*I restriction site polymorphism was associated with weight gain after a 100 day over-feeding period. Other studies (Merriwether et al. 1995; Rowe et al. 1997) also suggest that mtDNA polymorphisms play a pivotal role in obesity.

Understanding the association between mtDNA polymorphisms and obesity may be helpful in preventing obesity-related chronic diseases. We detected a novel mtDNA nucleotide variation: polymorphism of mtDNA 15497 guanine/adenine (Mt15497G→A). Although Mt15497G→A leads to the Gly251Ser amino acid replacement on human cytochrome *b*, it is unknown whether a functional alteration of the mitochondrion is induced by the Gly251Ser replacement (Tanaka et al. 2002). Therefore, to elucidate an association between the Mt15497G→A polymorphism and obesity, we examined a relatively large sample size of

middle-aged to elderly Japanese individuals comparing body size, body composition, and regional body fat distribution of subjects carrying the G or A alleles of Mt15497.

Materials and methods

Subjects

The subjects were 825 women and 906 men who participated in the 2nd wave of examinations in the National Institute for Longevity Sciences-Longitudinal Study of Aging (NILS-LSA) from April 2000 to April 2002. These were randomly sampled, community-dwelling individuals aged 42 –82 years, stratified by age and gender, living in the neighborhood of the NILS. Details of the NILS-LSA have been described elsewhere (Shimokata et al. 2000). Physical characteristics of subjects are shown in Table 1. The aim and design of the study was explained to each subject before they gave their written informed consent. The study protocol was approved by the Committee on Ethics of Human Research of National Chubu Hospital and the NILS.

Determination of Mt15497 genotype

Mt15497 genotype was determined with an automated colorimetric allele-specific DNA probe assay system (Toyobo Gene Analysis, Tsuruga, Japan). In brief, the polymorphic region of the gene was amplified by polymerase chain reaction (PCR) with allele-specific sense (5′-TATTCTCACCAGACCTCCTXGG-3′ and 5′-ACTATTCTCACCAGACCTCCTXAG-3′) and biotin-labeled antisense (5′-GTGTTTAAGGGGTTGGCTAGG-3′) primers. The reaction mixture (25 µl) contained 50 ng of DNA, 5 pmol of each primer, 0.2 mmol/l of each deoxynucleoside triphosphate, 2.5 mmol/l $MgCl_2$, and 1 U of 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 60°C for 30 s, and extension at 72°C for 30 s; and a final extension at 72°C for 2 min. Amplified DNA was denatured with 0.3 M NaOH and then subjected to hybridization at 37°C for 30 min in hybridization buffer containing 35% formamide with allele-specific capture probes (5′- TCCTXGGCGACCCAGACAA-3′ or 5′- CTCCTXAGCGACCCAGACAAT-3′) fixed to the bottom of the wells of a 96-well plate. After thorough washing of the wells, alkaline phosphatase-conjugated streptavidin was added to each well and the plate was incubated at 37°C for 15 min with agitation. The wells were again washed, and after the addition of a solution containing 0.8 mmol/l 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (monosodium salt) and 0.4 mmol/l 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt, absorbance at 450 nm was measured.

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 body mass index (BMI) was calculated as weight (kg) divided by height squared (m^2). Waist circumference and waist-to-hip ratio were used as the indices for body fat distribution in this study. The waist-to-hip ratio was calculated as a ratio of waist circumference measured at the level of the umbilicus to hip circumference.

Table 1 Descriptive characteristics of subjects (n=1731)

Variables	Women	Men
Number of subjects	825	906
Age (year)	60.1±0.4	60.8±0.3
Height (cm)	151.7±0.2	164.6±0.2
Body weight (kg)	52.6±0.3	62.7±0.3
Body mass index (kg/m^2)	22.9±0.1	23.1±0.1
Percentage fat mass (%)	30.8±0.2	21.3±0.1

Body composition by dual-energy x-ray absorptiometry

Whole-body fat mass, fat-free mass, and percentage fat mass assessed by dual-energy x-ray absorptiometry (QDR-4500, Hologic, Madison, OH, USA) were used as the indices for determining body composition. Transverse scans were used to measure fat mass and fat-free mass, and pixels of soft tissue were used to calculate the ratio (R value) of mass attenuation coefficients at 40–50 keV (low energy) and 80–100 keV (high energy) using software version 1.3Z.

Abdominal adipose tissue area by CT

The intra-abdominal fat area (IFA) and subcutaneous fat area (SFA) were measured at the level of the umbilicus (L4-L5) using computed tomography (CT) scans (SCT-6800TX, Shimadzu, Tokyo, Japan) carried out on subjects in the supine position. The IFA and SFA were calculated using a computer software program (FatScan, N2system, Osaka, Japan) (Yoshizumi et al. 1996). Firstly, a region of the SF layer was defined by tracing its contour on each scan, and the range of CT values (in Hounsfield units) for fat tissue was calculated. Total fat area was determined by delineating the surface having a mean CT value plus or minus two standard deviations, and the IFA was measured by drawing a line within the muscle wall surrounding the abdominal cavity. The SFA was then calculated by subtracting the IFA from the total fat area, and the IFA to SFA (I/S) ratio was determined. The intra-class correlation for repeated IFA determinations in our laboratory is 0.99.

Biochemical examination of blood

An antecubital blood sample was drawn from each subject after an overnight fast. Serum total cholesterol and triglycerides were determined enzymatically, serum high-density lipoprotein cholesterol was measured by the heparin-manganese precipitation method and fasting plasma glucose was assayed by a glucose oxidase method. Plasma insulin was measured in duplicate by radioimmunoassay. Glycosylated hemoglobin (HbA_{1c}) was measured by high performance liquid chromatography. Serum low-density lipoprotein cholesterol was estimated according to the Friedewald formula (1972).

Data analysis

Values are expressed as mean ± standard error (SE) in the tables. Quantitative data were compared by General Linear Model with age and smoking as covariates. Qualitative data were analyzed by the chi-square test. In each statistical analysis, probability values below 0.05 were regarded as significant. The data were analyzed with the Statistical Analysis System (SAS), version 8.2.

Results

The Mt15497G→A polymorphism was detected in 3.5% (n=60) of all subjects: 2.8% (n=23) of the women and 4.1% (n=37) of the men. In both sexes, age, smoking status, and height were similar between the Mt15497G→A genotypes (Table 2). To examine the gender's influence on the relationship between the Mt15497G→A genotypes

Table 2 Anthropometric variables, body composition and abdominal adipose tissue areas of subjects according to sex and Mt 15497G→A genotype

	Women			Men		
	G	A	*p* value	G	A	*p* value
Variables						
Number (%)	802(97.2)	23(2.8)	-	869(95.9)	37(4.1)	-
Age (year)	60.1±0.4	61.3±2.2	0.565	60.7±0.4	62.1±1.7	0.430
Smoking (%)	7.0	8.7	0.685	36.7	43.2	0.373
Anthropometric variables						
Height (cm)	152±0.3	153±1.2	0.462	165±0.2	166±1.1	0.263
Body weight (kg)	52.6±0.3	57.5±1.7	0.004	62.6±0.3	64.2±1.5	0.318
Body mass index (kg/m^2)	22.8±0.1	24.8±0.7	0.004	23.1±0.1	23.4±0.5	0.530
Waist circumference (cm)	83.0±0.3	88.0±2.0	0.014	85.0±0.3	87.4±1.3	0.087
Hip circumference (cm)	90.8±0.2	94.3±1.1	0.002	92.2±0.2	92.7±0.8	0.524
Waist to hip ratio	0.91±0.003	0.93±0.02	0.254	0.92±0.01	0.94±0.01	0.032
Body composition by dual-energy x-ray absorptiometry						
Percentage fat mass (%)	30.8±0.2	32.4±1.1	0.139	21.3±0.2	22.0±0.7	0.312
Fat mass (kg)	16.4±0.2	18.7±1.0	0.025	13.5±0.1	14.3±0.7	0.273
Fat-free mass (kg)	36.1±0.2	38.6±1.0	0.010	49.0±0.2	49.9±1.1	0.445
Abdominal adipose tissue area by computed tomography						
Intra-abdominal fat-area (cm^2)	63.7±1.5	84.4±8.6	0.017	93.7±1.7	110±8.5	0.065
Subcutaneous fat area (cm^2)	165±2.4	182±13.9	0.237	112±1.6	114±7.9	0.862
I/S ratio	0.40±0.01	0.48±0.05	0.122	0.86±0.01	0.99±0.07	0.062

I/S ratio: ratio of intra-abdominal and subcutaneous adipose tissue area. Data were adjusted for age and smoking

Table 3 Biochemical measurements according to sex and Mt 15497G→A genotype

	Women			Men		
	G	A	*p* value	G	A	*p* value
Variables						
Total cholesterol (mg/dl)	223.1±1.2	217.8±7.1	0.462	210.9±1.7	214.4±5.6	0.540
Triglycerides (mg/dl)	105.4±2.3	151.5±13.1	0.001	128.2±2.9	152.0±13.6	0.087
LDL cholesterol (mg/dl)	135.3±1.1	126.8±6.4	0.190	127.3±1.1	127.7±5.3	0.952
HDL cholesterol (mg/dl)	66.5±0.5	60.7±3.2	0.074	57.6±0.5	56.3±2.4	0.599
Glucose (mg/dl)	98.9±0.8	101.8±4.4	0.522	105.4±0.8	104.5±3.8	0.802
HbA_{1c} (%)	5.3±0.03	5.3±0.16	0.807	5.4±0.03	5.4±0.15	0.597
Insulin (μU/ml)	8.0±0.2	9.6±1.3	0.223	8.2±0.3	8.9±1.4	0.653

Data were adjusted for age and smoking

and anthropometric variables, body composition, abdominal adipose tissue areas, and biochemical blood parameters, we analyzed the data for men and women independently.

Anthropometric variables

For women, body weight, BMI, and waist and hip circumferences were significantly greater in subjects with the A allele than in those with the G allele (p=0.002–0.014). For men, waist to hip ratio was significantly greater in subjects with the A allele than in those with the G allele (p=0.032) and a trend toward significant difference was found in waist circumference (p=0.087). Although statistical significance was not achieved in any other variables (p>0.05), all measurement values were greater in subjects with the A allele than in those with the G allele.

Body composition

For women, both fat mass and fat-free mass were significantly greater in subjects with the A allele than in those with the G allele (p=0.025 and 0.010, respectively). For men, although no significant difference was found in any measurement variables between the genotypes, all measurement values were greater in subjects with the A allele than in those with the G allele.

Abdominal adipose tissue area

For women, IFA was significantly greater in subjects with the A allele than in those with the G allele (p=0.017). For men, a trend toward significant difference was found in IFA (p=0.065) and I/S ratio (p=0.062).