We next examined the relationship of the Mt15497G→A polymorphism with biochemical parameters for lipid and glucose metabolism (Table 3). For women, triglycerides were significantly higher in subjects with the A allele than in those with the G allele (p=0.001). Plasma insulin was also 20% higher in subjects with the A allele compared with the G allele, but statistical significance was not achieved. For men, a trend toward significant difference was found in triglycerides (p=0.087).

Discussion

The mitochondrial oxidative phosphorylation system is a major source of energy utilization for cellular activities. Therefore, we tested a hypothesis that an association exists between the Mt15497 polymorphism and obesity-related measurement variables by examining a relatively large sample size of middle-aged to elderly Japanese men and women. Our data revealed that several obesity-related variables were significantly different between subjects having either the G or A alleles of Mt15497. It may be that increased efficiency of mitochondrial energy conservation at the cytochrome *bc*1 complex results in decreased energy consumption (Tanaka et al.2002). Another possibility is that inhibiting reduction in ubiquinone at the Qo site (one of the ubiquinone-binding sites of complex III) results in a reduced β-oxidation of fatty acid, which leads to fat accumulation. We are currently constructing cybrid clones carrying the Mt15497G→A polymorphism to determine whether the G251S replacement results in decreased activity of ubiquinol-cytochrome *c* reductase (complex III) or not. Instead, we carried out a molecular dynamic simulation to understand the effect of the G251S replacement to the molecular structure of cytochrome *b* in our database system http://www.giib.or.jp/mtsnp/search_mtSAP_3D_e.html. Andreu et al. argued that the G251D replacement due to the Mt15498G→A mutation is pathogenic because the presence of Asp instead of Gly should cause charge repulsion with Glu271, a residue at the Qp site (Andreu et al. 2000). This, in turn, would change the structure of the Qp site and impair hydroquinone binding.

For women, anthropometric variables, body composition and abdominal adipose tissue area were significantly associated with the Mt15497 polymorphism, whereas these associations were weaker in men compared with women. There is other evidence supporting the idea that the association of genetic variation with obesity is stronger in women than men (Borecki et al. 1993; Comuzzie et al. 1995). For instance, Comuzzie et al. (1995), in a Mendelian mixed model analysis for fat mass, incorporating genotype by gender interaction, reported that the major gene accounted for 37% of the total variance of fat mass in men compared with 43% in women. To our knowledge, however, little has been reported on gender's effect on the association between a mtDNA polymorphism and obesity.

It is well known that waist size and the amount of intra-abdominal adipose tissue are strongly associated with various risk factors for coronary heart disease [e.g., hypertension (Kanai et al. 1990; Matsuzawa et al. 1995), Type 2 diabetes (Yamashita et al. 1996; Macor et al. 1997) and Type 1 plasminogen activator inhibitor (Svendsen et al. 1996; Lindahl et al. 1998)]. Japan Society for the Study of Obesity (2002) defined "obesity disease" as not only the presence of obesity-related complications, but also their likely occurrence. In this case, "likely occurrence" means high-risk obesity as specified by an excess IFA (greater than 100 cm^2) measured by CT scan. Thus, individuals with high-risk obesity have a strong chance of suffering from obesity-related complications in the near future.

The results presented in Table 3 show a significant association in women and a trend toward significant association in men between high triglycerides levels and the Mt15497 polymorphism. In addition, plasma insulin level was 20% higher in women with the A allele compared to those with the G allele (see Table 3). Kokaze et al. (2001) found in their epidemiological study that the Mt5178A/C polymorphism was associated with the triglycerides level in Japanese women, and Gerbitz (1992) reported that impairment of ATP production by a mtDNA mutation caused insulin secretion defects and possibly insulin resistance as well. These reports suggest that mtDNA polymorphisms impair lipid metabolism and insulin secretion through a defect of mitochondrial function.

Detailed causes of the significant difference found in fat-free mass between the Mt15497 genotypes in women could not be clarified. Keightley et al. compared 10 cases of cytochrome *b* mutation and found that most of the patients in those studies presented with the predominant feature of severe exercise intolerance or hypertrophic cardiomyopathy (Keightley et al. 2000). Andreu et al. reported that the G251D replacement due to the Mt15498G→A mutation led to heart failure (histiocytoid cardiomyopathy) (Andreu et al. 2000). However, it is unknown how the Gly251Ser replacement by the Mt15497G→A mutation affects the human body. Finding of larger fat-free mass in subjects with the A allele of Mt15497 in our study is likely to indicate that increased fat mass indirectly affects the fat-free mass.

In conclusion, we have shown in a relatively large sample of middle-aged to elderly Japanese that significant associations exist between the Mt15497 polymorphism and body size, body composition, abdominal adipose tissue area, and lipid metabolism. Although these data suggest that the A allele of Mt15497 may be one of the important determinants of obesity, further studies are needed to validate our speculation.

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Association of polymorphisms of the osteoprotegerin gene with bone mineral density in Japanese women but not men

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Abstract

Given that osteoprotegerin plays an important role in bone remodeling, the osteoprotegerin gene may be a candidate locus for susceptibility to osteoporosis. The relation of polymorphisms in the promoter of the osteoprotegerin gene to bone mineral density (BMD) was examined in a Japanese population-based prospective cohort study with randomly recruited subjects (1095 women and 1125 men for the 950T → C polymorphism, 1094 women and 1127 men for the 245T → G polymorphism). BMD at the radius was measured by peripheral quantitative computed tomography, and that for the total body, lumbar spine, right femoral neck, right trochanter, and right Ward's triangle was measured by dual-energy X-ray absorptiometry. Genotypes were determined with a fluorescence-based allele-specific DNA primer assay system. Among 950T → C genotypes, BMD for the proximal radius was lower in premenopausal women with the *CC* genotype than in those with the *TT* or *TC* genotype; the difference in BMD between the two groups was 3.9% ($P = 0.0075$). Among 245T → G genotypes, BMD for the radius, total body, femoral neck, trochanter, and Ward's triangle was lower in postmenopausal women with the *GG* genotype than in those with the *TT* or *TG* genotype, the *TT* genotype, or the *TG* genotype; the differences in BMD between the *GG* genotype and the *TT* or *TG* genotype were 19.8% for the distal radius ($P = 0.0015$), 13.1% for the proximal radius ($P = 0.0095$), 11.2% for the total body ($P = 0.0013$), 12.9% for the femoral neck ($P = 0.0067$), 18.7% for the trochanter ($P = 0.0008$), and 27.1% for Ward's triangle ($P = 0.0038$). BMD was not associated with the 950T → C or 245T → G genotypes in men. The present results implicate the osteoprotegerin gene as a susceptibility locus for reduced BMD in Japanese women.
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Keywords: Bone mineral density; Genetics; Osteoporosis; Osteoprotegerin; Osteoclastogenesis inhibitory factor; Polymorphism

Introduction

Osteoporosis, a major health problem of the elderly, is characterized by a reduction in bone mineral density (BMD) and a deterioration in the microarchitecture of bone, both of which result in predisposition to fractures [1]. Although several environmental factors, including diet, smoking, and physical exercise, influence BMD, a genetic contribution to this parameter has been recognized [2]. Genetic linkage analyses [3–5] and candidate gene association studies [5–7] have thus implicated several loci and candidate genes in the regulation of bone mass and the prevalence of osteoporosis or osteoporotic fractures. The genes that contribute to genetic susceptibility to osteoporosis, however, remain to be fully characterized.

Osteoprotegerin is a soluble member of the tumor necrosis factor (TNF) receptor superfamily of proteins. In vitro studies suggest that osteoprotegerin inhibits osteoclastogenesis by interrupting intercellular signaling between osteoblastic stromal cells and osteoclast progenitors [8]. Osteoprotegerin-deficient mice exhibit a condition similar to juvenile Paget's disease characterized by a marked decrease in trabecular and cortical bone density, pronounced thinning of the parietal bone of the skull, and a high incidence of fractures [9], whereas hepatic expression of osteoprotegerin in transgenic mice results in osteopetrosis and a coincident decrease in the proportion of osteoclasts at the later stages

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of differentiation [8]. The systemic administration of recombinant osteoprotegerin also results in a marked increase in BMD in normal rats as well as in prevention of bone loss in ovariectomized rats [8,10]. Furthermore, a single subcutaneous injection of osteoprotegerin reduced bone resorption in postmenopausal women [11]. Similar treatment with a recombinant osteoprotegerin construct (AMGN-0007) suppressed bone resorption in patients with multiple myeloma or breast cancer with bone metastases [12]. Given the importance of osteoprotegerin in bone remodeling, the osteoprotegerin gene (*OPG*) may be a candidate locus for susceptibility to osteoporosis.

Several single nucleotide polymorphisms (SNPs) have been detected in *OPG*, some of which have been shown to be associated with BMD in postmenopausal women [13–15] or with osteoporotic fractures in women and men [16]. Given the ethnic divergence of gene polymorphisms, however, it is important to examine polymorphisms potentially related to BMD in each ethnic group. We have now examined whether the 950T → C and 245T → G SNPs in the promoter of *OPG* are associated with BMD in Japanese women or men in a population-based study.

Materials and methods

Study population

The present study represents a cross-sectional analysis in a population-based prospective cohort study of aging and age-related diseases [17,18]. The subjects 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 in this area is typical of that of individuals in most regions of Japan. The numbers of men and women recruited are similar and the baseline age is 40–79 years, with similar numbers of participants in each decade (40s, 50s, 60s, and 70s). The subjects will be followed up every 2 years. 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. Individuals with disorders known to cause abnormalities of bone metabolism, including diabetes mellitus, renal diseases, rheumatoid arthritis, as well as thyroid, parathyroid, and other endocrinologic diseases, were excluded from the present study. Women who had taken drugs, such as estrogen, progesterone, glucocorticoids, and bisphosphonates were also excluded. We examined the relation of BMD at various sites to the 950T → C SNP of *OPG* in 2220 participants (1095 women, 1125 men) and to the 245T → G SNP in 2221 participants (1094 women, 1127 men). The study protocol was approved by the relevant committee on the ethics of human research and written informed consent was obtained from each subject.

Measurement of BMD

BMD at the radius was measured by peripheral quantitative computed tomography (pQCT) (Desiscan 1000; Scanco Medical, Bassersdorf, Switzerland) and was expressed as D50 (distal radius BMD for the inner 50% of the cross-sectional area, comprising mostly cancellous bone), D100 (distal radius BMD for the entire cross-sectional area, including both cancellous and cortical bone), and P100 (proximal radius BMD for the entire cross-sectional area, consisting mostly of cortical bone). BMD for the 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, 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 genotypes

Genotypes were determined with a fluorescence-based allele-specific DNA primer assay system (Toyobo Gene Analysis, Tsuruga, Japan) [19]. The polymorphic regions of *OPG* were amplified by the polymerase chain reaction with allele-specific sense primers labeled at the 5′ end with either fluorescein isothiocyanate (5′-CAGCCCTGAAAGCGTTAXTC-3′ for 950T → C and 5′-CGCTGAACTTCTGGAGXAG-3′ for 245T → G) or Texas red (5′-CAGCCCTGAAAGCGTTAXCC-3′ for 950T → C and 5′-CGCTGAACTTCTGGAGXCG-3′ for 245T → G) and with an antisense primer labeled at the 5′ end with biotin (5′-GGGTGTGCAGAAAGCTCCA-3′ for 950T → C and 5′-GCTTGAGGCTAGTGGAAAGAC-3′ for 245T → G). The reaction mixtures (25 μL) contained 20 ng DNA, 5 pmol each primer, 0.2 mmol/L each deoxynucleoside triphosphate, 2.5 mmol/L $MgCl_2$, and 1 U 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 s, annealing at 62.5 °C (950T → C) or 60 °C (245T → G) for 30 s, and extension at 68 °C for 30 s; and a final extension at 68 °C for 2 min.

The 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 then placed on a magnetic stand and 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.

Statistical analysis

Quantitative data 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. Allele frequencies were estimated by the gene-counting method, and the χ^2 test was used to identify significant departure from Hardy–Weinberg equilibrium. Unless indicated otherwise, a P value of <0.05 was considered statistically significant.

Results

The distribution of combined genotypes for the 950T→C and 245T→G SNPs of *OPG* is shown in Table 1. The distribution of haplotypes was as follows: *950T/245T*, 56.1%; *950T/245G*, 4.1%; *950C/245T*, 32.9%; and *950C/245G*, 6.9%. The two SNPs exhibited marked linkage disequilibrium [pairwise linkage disequilibrium coefficient, D' (D/D_{max}), of 0.3858 and standardized linkage disequilibrium coefficient, r, of 0.1664; $P < 0.0001$].

To examine the possible influence of menopause on the relation between genotype and BMD, we analyzed BMD and other characteristics for premenopausal and postmenopausal women independently. The distributions of 950T→C genotypes of *OPG* were in Hardy–Weinberg equilibrium for both premenopausal and postmenopausal women (Table 2). Age for premenopausal or postmenopausal women and years since menopause for postmenopausal women did not differ among 950T→C genotypes. Given the multiple comparisons of genotypes with BMD values, we considered a P value of <0.01 to be significant for such associations. On the basis of this criterion, for premenopausal women, BMD at the proximal radius (P100) was significantly lower in those with the *CC* genotype than in those with the *TT* or *TC* genotype. The difference in P100 between the *CC* genotype and the *TT* or *TC* genotype (expressed as a percentage of the corresponding larger value) in premenopausal women was 3.9% ($P = 0.0075$). In contrast, for postmenopausal women, BMD was not associated with 950T→C genotype (Table 2). For men, the distribution of 950T→C ge-

Table 1
Distribution of the 950TC and 245T→G genotypes of *OPG* among study subjects

245T→G genotype	950T→C genotype			Total
	TT	*TC*	*CC*	
TT	804 (36.05%)	785 (35.20%)	193 (8.65%)	1782 (79.91%)
TG	0 (0%)	284 (12.74%)	135 (6.05%)	419 (18.79%)
GG	0 (0%)	0 (0%)	29 (1.30%)	29 (1.30%)
Total	804 (36.05%)	1069 (47.94%)	357 (16.01%)	2230 (100%)

Table 2
BMD and other characteristics of women ($n = 1095$) according to menopausal status and the 950T→C genotype of *OPG*

Characteristic	Premenopausal women ($n = 279$)				Postmenopausal women ($n = 816$)			
	TT	*TC*	*TT* + *TC*	*CC*	*TT*	*TC*	*TT* + *TC*	*CC*
Number (%)	101 (36.2)	131 (47.0)	232 (83.2)	47 (16.8)	295 (36.2)	387 (47.4)	682 (83.6)	134 (16.4)
Age (years)	46.1 ± 0.5	46.3 ± 0.4	46.2 ± 0.3	46.1 ± 0.7	64.3 ± 0.5	63.8 ± 0.4	64.0 ± 0.3	63.7 ± 0.7
Years since menopause					15.4 ± 0.5	14.7 ± 0.5	15.0 ± 0.4	14.7 ± 0.8
BMD values measured by pQCT (mg/cm³)								
D50	248.2 ± 5.8	245.7 ± 5.2	246.8 ± 3.8	237.5 ± 8.4	163.3 ± 4.1	162.4 ± 3.6	162.8 ± 2.7	166.8 ± 6.1
D100	606.3 ± 8.1	609.0 ± 7.3	607.8 ± 5.4	591.8 ± 11.8	436.9 ± 6.4	444.1 ± 5.6	441.0 ± 4.2	451.7 ± 9.6
P100	1368.4 ± 12.5*	1369.5 ± 11.2*	1369.0 ± 8.3†	1315.2 ± 18.2	1064.0 ± 11.1	1085.0 ± 9.8	1075.9 ± 7.3	1093.7 ± 16.6
BMD values measured by DXA (g/cm²)								
Total body	1.084 ± 0.009	1.093 ± 0.008	1.090 ± 0.006	1.113 ± 0.013	0.917 ± 0.006	0.920 ± 0.006	0.919 ± 0.004	0.919 ± 0.010
L2–L4	1.018 ± 0.012	1.026 ± 0.011	1.023 ± 0.008	1.033 ± 0.018	0.802 ± 0.009	0.813 ± 0.008	0.808 ± 0.006	0.815 ± 0.013
Femoral neck	0.771 ± 0.010	0.767 ± 0.009	0.769 ± 0.007	0.788 ± 0.014	0.641 ± 0.006	0.644 ± 0.005	0.643 ± 0.004	0.650 ± 0.009
Trochanter	0.656 ± 0.009	0.654 ± 0.008	0.655 ± 0.006	0.674 ± 0.013	0.540 ± 0.006	0.537 ± 0.005	0.538 ± 0.004	0.547 ± 0.009
Ward's triangle	0.650 ± 0.013	0.659 ± 0.011	0.655 ± 0.009	0.680 ± 0.019	0.442 ± 0.009	0.457 ± 0.008	0.451 ± 0.006	0.462 ± 0.013

Data are means ± SE. pQCT, peripheral quantitative computed tomography; DXA, dual-energy X-ray absorptiometry.
* $P < 0.05$.
† $P < 0.01$ versus *CC*.

notypes was in Hardy–Weinberg equilibrium, but BMD did not differ among these genotypes (data not shown).

The distributions of genotypes for the 245T → G SNP of *OPG* were in Hardy–Weinberg equilibrium in both premenopausal and postmenopausal women (Table 3). Age did not differ among 245T → G genotypes for premenopausal women. For postmenopausal women, age and years since menopause were greater in individuals with the *GG* genotype than in those with the *TT* or the *TG* genotypes. For premenopausal women, BMD was not associated with 245T → G genotype. On the basis of the criterion of $P < 0.01$, for postmenopausal women, BMD for the distal (D100) or proximal (P100) radius, total body, femoral neck, trochanter, or Ward's triangle was significantly lower in those with the *GG* genotype than in those with the *TT* or *TG* genotype, with the *TT* genotype, or with the *TG* genotype (Table 3). The differences in BMD between the *GG* genotype and the *TT* or *TG* genotype in postmenopausal women were 19.8% for D100 ($P = 0.0015$), 13.1% for P100 ($P = 0.0095$), 11.2% for the total body ($P = 0.0013$), 12.9% for the femoral neck ($P = 0.0067$), 18.7% for the trochanter ($P = 0.0008$), and 27.1% for Ward's triangle ($P = 0.0038$). For men, the distribution of genotypes for the 245T → G SNP was in Hardy–Weinberg equilibrium, but BMD did not differ among these genotypes (data not shown).

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 [17]. We also showed that the genotype distributions for both of the SNPs studied were in Hardy–Weinberg equilibrium both in women and in men. Our study population therefore appeared genetically homogeneous, and we thus appeared to avoid admixture and selection bias.

Osteoclastogenesis is regulated by three TNF- or TNF receptor-related proteins: receptor activator of nuclear factor-κB (RANK) [20,21], RANK ligand (RANKL) [22,23], and osteoprotegerin [8,10]. RANKL expressed on the surface of bone marrow stromal cells induces the differentiation of osteoclasts, enhances the activity of mature osteoclasts, and inhibits osteoclast apoptosis by binding to its functional receptor, RANK, expressed on osteoclasts or their progenitors [22–26]. The interaction between RANKL and RANK is antagonized by osteoprotegerin, which acts as a decoy receptor for RANKL. The biological effects of osteoprotegerin include inhibition of the later stages of osteoclastogenesis [8,10,27], suppression of the activation of mature osteoclasts [22,25,28], and induction of osteoclast apoptosis [29]. The balance between osteoprotegerin and RANKL may thus represent an important determinant of bone resorption [27]. The importance of osteoprotegerin in the regulation of bone remodeling in humans has been indicated by the occurrence of juvenile Paget's disease, characterized by rapid remodeling of

Table 3
BMD and other characteristics of women ($n = 1094$) according to menopausal status and the 245T → G genotype of *OPG*

Characteristic	Premenopausal women ($n = 276$)				Postmenopausal women ($n = 818$)			
	TT	*TG*	*TT + TG*	*GG*	*TT*	*TG*	*TT + TG*	*GG*
Number (%)	225 (81.5)	49 (17.8)	274 (99.3)	2 (0.7)	637 (77.9)	169 (20.7)	806 (98.5)	12 (1.5)
Age (years)	46.2 ± 0.3	46.1 ± 0.7	46.2 ± 0.3	49.0 ± 3.2	63.9 ± 0.3*	63.7 ± 0.7*	63.9 ± 0.3*	70.1 ± 2.5
Years since menopause					14.9 ± 0.4*	14.9 ± 0.7*	14.9 ± 0.3*	20.9 ± 2.7
BMD values measured by pQCT (mg/cm³)								
D50	244.4 ± 3.9	248.1 ± 8.3	245.1 ± 3.5	280.0 ± 40.4	164.0 ± 2.8*	164.6 ± 5.4*	164.1 ± 2.5*	115.6 ± 19.8
D100	605.3 ± 5.5	605.5 ± 11.8	605.3 ± 5.0	618.0 ± 57.1	443.6 ± 4.4*	446.3 ± 8.5*	444.2 ± 3.9†	356.4 ± 31.1
P100	1360.1 ± 8.6	1358.7 ± 18.3	1359.9 ± 7.8	1334.0 ± 88.7	1081.6 ± 7.6*	1078.4 ± 14.7*	1081.0 ± 6.7‡	939.5 ± 54.1
BMD values measured by DXA (g/cm²)								
Total body	1.093 ± 0.006	1.093 ± 0.012	1.093 ± 0.005	1.115 ± 0.061	0.923 ± 0.004‡	0.911 ± 0.009*	0.920 ± 0.004†	0.817 ± 0.032
L2–L4	1.024 ± 0.008	1.029 ± 0.018	1.024 ± 0.007	1.039 ± 0.088	0.809 ± 0.006	0.815 ± 0.012	0.810 ± 0.005*	0.715 ± 0.044
Femoral neck	0.772 ± 0.007	0.770 ± 0.014	0.772 ± 0.006	0.694 ± 0.070	0.644 ± 0.004*	0.647 ± 0.008*	0.645 ± 0.004‡	0.562 ± 0.030
Trochanter	0.658 ± 0.006	0.658 ± 0.013	0.658 ± 0.005	0.594 ± 0.063	0.543 ± 0.004†	0.535 ± 0.008‡	0.541 ± 0.004§	0.440 ± 0.030
Ward's triangle	0.660 ± 0.009	0.656 ± 0.019	0.659 ± 0.008	0.593 ± 0.092	0.455 ± 0.006‡	0.454 ± 0.011*	0.454 ± 0.005†	0.331 ± 0.042

Data are means ± SE. pQCT, peripheral quantitative computed tomography; DXA, dual-energy X-ray absorptiometry.
* $P < 0.05$.
† $P < 0.005$.
‡ $P < 0.01$.
§ $P < 0.001$ versus *GG*.

woven bone, osteopenia, fractures, and progressive skeletal deformity, in Navajo individuals homozygous for a deletion of ~100 kb in *OPG* [30].

Previously described SNPs in *OPG* include 163A → G, 209G → A, 245T → G, 889C → T, 950T → C, 1181G → C, and 6890A → C [13–16]. Of these polymorphisms, we selected 950T → C, which is identical to the −223T → C SNP [13], because it was previously shown to be associated with radial BMD in postmenopausal Japanese women, with the *TT* genotype representing a risk factor for reduced BMD [13]. We chose the 245T → G SNP because it was previously associated with the prevalence of osteoporosis and vertebral fracture [16]. We have now shown that the 950T → C SNP was associated with BMD for the proximal radius in premenopausal Japanese women, with the *CC* genotype representing a risk factor for reduced BMD. The reason for the discrepancy between our results and those of the previous study of this polymorphism [13] is unclear, but there are several differences between the two studies. The skeletal sites and methods of BMD measurement thus differed; whereas BMD for total radial bone, including both cancellous and cortical bone, was measured by DXA in the previous study [13], BMD for the proximal radius, including mostly cortical bone, was measured by pQCT in our study. Age and menopausal state of subjects also differed between the two studies; the subjects were postmenopausal women (mean age, 73.2 years) in the previous study [13], whereas the subjects were premenopausal women (mean age, 46.2 years) in our study. Given that the 950T → C SNP is located in the promoter region of *OPG* and was associated with BMD in premenopausal, but not postmenopausal, women in our study, we hypothesize that this polymorphism might affect the promoter activity of *OPG* and that this effect may be estrogen-dependent.

Both 209G → A and 245T → G SNPs of the *OPG* promoter have previously been associated with BMD for the lumbar spine in postmenopausal Slovenian women, with the *209GA/245TG* genotype representing a risk factor for reduced BMD [14]. The 163A → G and 245T → G SNPs were also associated with vertebral fractures in Danish women and men, with the *G* allele of each SNP representing a risk factor for fracture [16]. We have now shown that the 245T → G SNP was associated with BMD for the radius, total body, femoral neck, trochanter, and Ward's triangle in postmenopausal Japanese women, with the *GG* genotype representing a risk factor for reduced BMD, consistent with these previous observations [14,16]. However, the distribution of 245T → G genotypes was significantly different between the studies of Arko et al. [14] (*TT*, 89.3%; *TG*, 10.7%; *GG*, 0%; $P = 0.0328$, χ^2 test) or Langdahl et al. [16] (*TT*, 90.3%; *TG*, 9.2%; *GG*, 0.5%; $P < 0.0001$) and our study (women: *TT*, 78.8%; *TG*, 19.9%; *GG*, 1.3%). These differences in genotype distribution may be attributable to ethnic differences.

The molecular mechanisms that underlie the association of the 950T → C or 245T → G SNPs of *OPG* with BMD remain unclear. The effects of these SNPs on the transcriptional activity of the *OPG* promoter have not been determined. It is possible that the SNPs examined in our study are in linkage disequilibrium with polymorphisms of other nearby genes that are actually responsible for the association with BMD.

In conclusion, the 245T → G SNP of *OPG* was associated with BMD at various sites in postmenopausal Japanese women in the present study, consistent with its previously demonstrated associations with the prevalence of osteoporosis and vertebral fracture in Danish women [16] and with BMD for the lumbar spine in postmenopausal Slovenian women [14]. The 950T → C SNP was also associated with BMD for the proximal radius in premenopausal Japanese women in the present study, although the effect of this SNP on BMD was relatively small. Our present results thus suggest that *OPG* is a candidate locus for reduced bone mass in women.

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ORIGINAL ARTICLE

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Association of polymorphisms of paraoxonase 1 and 2 genes, alone or in combination, with bone mineral density in community-dwelling Japanese

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Abstract Oxidative stress may affect cellular functions in various pathological conditions, including osteoporosis. Paraoxonase 1 confers antioxidant properties on high-density lipoprotein, with which it is associated, by reducing the accumulation of lipid peroxidation products. We have now examined whether the 584A → G (Gln192Arg) and 172T → A (Leu55Met) polymorphisms of the paraoxonase 1 gene and the 959G → C (Cys311Ser) polymorphism of the paraoxonase 2 gene are associated with bone mineral density (BMD) in community-dwelling Japanese (1,087–1,094 women and 1,112–1,125 men). The subjects were aged 40 –79 years and were randomly recruited to a population-based prospective cohort study of aging and age-related diseases. BMD for the lumbar spine and right femoral neck was measured by dual-energy X-ray absorptiometry. Genotypes were determined with a fluorescence- or colorimetry-based allele-specific DNA primer-probe assay system. The 584A → G and 172T → A polymorphisms of the paraoxonase 1 gene and the 959G → C polymorphism of the paraoxonase 2 gene were associated with BMD for the lumbar spine or femoral neck in postmenopausal women, with the 584*GG*, 172*TT*, and 959*CC* genotypes representing risk factors for reduced bone mass. None of these three polymorphisms was associated with BMD in premenopausal women or in men. Our results suggest that the paraoxonase 1 and 2 genes are candidate loci for reduced bone mass in postmenopausal Japanese women.

Keywords Bone mineral density · Genetics · Osteoporosis · Paraoxonase · Polymorphism

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Introduction

Osteoporosis is characterized by a reduction in bone mineral density (BMD) and a deterioration in the microarchitecture of bone, both of which result in an increased susceptibility to fractures (Kanis et al. 1994). Although several environmental factors, such as diet and physical exercise, influence BMD, a genetic contribution to the etiology of osteoporosis has been recognized (Pocock et al. 1987). Genetic linkage analyses (Devoto et al. 1998; Johnson et al. 1997; Morrison et al. 1994) and candidate gene association studies (Morrison et al. 1994; Uitterlinden et al. 1998; Yamada et al. 2001) have thus implicated several loci and candidate genes in the regulation of bone mass and the pathogenesis of osteoporotic fractures. The genes that contribute to genetic susceptibility to osteoporosis, however, remain to be identified definitively.

Oxidative stress may affect cellular functions in various pathological conditions, including osteoporosis (Basu et al. 2001; Garrett et al. 1990). Recent evidence has suggested that lipid oxidation contributes to the development of osteoporosis (Parhami et al. 2000). In vitro studies indicate that lipid oxidation products promote osteoblastic differentiation of vascular cells and inhibit such differentiation of bone cells (Parhami et al. 1997). Oxidation products of low-density lipoprotein (LDL) also promote osteoporotic loss of bone by inducing progenitor marrow stromal cells to undergo adipogenic rather than osteogenic differentiation (Parhami et al. 1999).

Paraoxonase 1 (PON1) is a calcium-dependent esterase that is closely associated with high-density

lipoprotein (HDL)-containing apolipoprotein A-I and is thought to confer antioxidant properties on HDL by preventing the accumulation of lipid peroxidation products (Mackness et al. 1991). This property of PON1 accounts for its ability to protect against atherosclerosis. In addition to the PON1 gene (*PON1*), related genes designated *PON2* and *PON3* have been identified in the human genome and are linked with *PON1* on chromosome 7q21-q22 (Primo-Parmo et al. 1996). Two single nucleotide polymorphisms (SNPs), 584A → G (Gln192 Arg; GenBank accession no. M63012) and 172T → A (Leu55Met; accession no. M63012), of *PON1* and a 959G → C (Cys311Ser; accession no. L48513) SNP of *PON2* have been associated with coronary artery disease (Ruiz et al. 1995; Serrato and Marian 1995; Garin et al. 1997; Sanghera et al. 1998). Although PON1 catalyzes the reduction of oxidized LDL and thereby may affect bone remodeling, the possible relations of these SNPs to BMD have not been determined.

We have now examined whether the 584A → G (Gln192Arg) and 172T → A (Leu55Met) SNPs of *PON1* and the 959G → C (Cys311Ser) SNP of *PON2* are associated with BMD in women or men in a large-scale, population-based study.

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 (Shimokata et al. 2000). 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 the baseline age is 40–79 years, with similar numbers of participants in each decade (40s, 50s, 60s, 70s). The subjects will be followed up every 2 years.

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. Individuals with disorders known to cause abnormalities of bone metabolism, including diabetes mellitus, renal diseases, rheumatoid arthritis, as well as thyroid, parathyroid, and other endocrinologic diseases, were excluded from the study. Women who had taken drugs such as estrogen, progesterone, glucocorticoids, and bisphosphonates were also excluded.

We examined the possible association of BMD with the 584A → G (Gln192Arg) SNP of *PON1* in 2,199 participants (1,087 women, 1,112 men), with the 172T → A (Leu55Met) SNP of *PON1* in 2,210 participants (1,092 women, 1,118 men), and with the 959G → C (Cys311Ser) SNP of *PON2* in 2,219 participants (1,094 women, 1,125 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 for the lumbar spine (L2–L4) and right femoral neck was measured by dual-energy X-ray absorptiometry (DEXA) (QDR 4500; Hologic, Bedford, MA, USA). The coefficients of variance of the DEXA instrument were 0.9% for L2–L4 and 1.3% for the femoral neck.

Determination of genotypes

Genotypes were determined with a fluorescence- or colorimetry-based allele-specific DNA primer-probe assay system (Toyobo Gene Analysis, Tsuruga, Japan).

For determination of genotype of the 584A → G (Gln192Arg) SNP, the polymorphic region of *PON1* was amplified by the polymerase chain reaction (PCR) with a sense primer labeled at the 5' end with biotin (5'-GAATGATATTGTTGCTGTGGGACC-3') and allele-specific antisense primers (5'-AACCCAAATACATCTCCC-AGGAXTG-3' or 5'-ACCCAAATACATCTCCCAGGAXCG-3').

For determination of 172T → A (Leu55Met) genotype, the polymorphic region of *PON1* was similarly amplified with a sense primer (5'-TCTGGCAGAAACTGGCTCTGAA-3') and an antisense primer labeled at the 5' end with biotin (5'-GCTA-ATGAAAGCCAGTCCATTA-3').

For determination of 959G → C (Cys311Ser) genotype, the polymorphic region of *PON2* was amplified by PCR with allele-specific sense primers labeled at the 5' end with either fluorescein isothiocyanate (5'- CGCATCCAGAACATTCTAXGT-3') or Texas red (5'- CCGCATCCAGAACATTCTAXCT-3') and with an antisense primer labeled at the 5' end with biotin (5'-GGCA-TAAACTGTAGTCACTGTAGGC-3').

The reaction mixtures (25 μl) contained 20 ng of DNA, 5 pmol of each primer, 0.2 mmol/l of each deoxynucleoside triphosphate, 1.4 mmol/l $MgSO_4$ (584A → G and 172T → A genotypes) or 1.8 mmol/l $MgCl_2$ (959G → C genotype), and 1 U of 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 cycles (584A → G and 959G → C genotypes) or 40 cycles (172T → A genotype) of denaturation at 95°C for 30 s, annealing at 63°C (584A → G genotype), 55°C (172T → A genotype), or 60°C (959G → C genotype) for 30 s, and extension at 68°C for 30 s; and a final extension at 68°C for 2 min.

For determination of 584A → G and 172T → A genotypes, amplified DNA was denatured with 0.3 mol/l NaOH and then subjected to hybridization at 37°C for 30 min in hybridization buffer containing 35% (584A → G genotype) or 32.5% (172T → A genotype) formamide with allele-specific capture probes (5'-ACATCTCCCAGGAXTGTAAGTAG-3' or 5'-ACA-TCTCCCAGGAXCGTAAGTAG-3' for 584A → G genotype, and 5'-GAAGACTTGGAGATACTGCC-3' or 5'-GAAGACATG-GAGATACTGCC-3' for 172T → A 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 again washed, and after the addition of a solution containing 0.8 mmol/l 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium (monosodium salt) and 0.4 mmol/l 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt, absorbance at 450 nm was measured.

For determination of 959G → C 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/l 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.

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

Assay for serum activity of PON1

Venous blood was collected from subjects after an overnight fast. Blood samples were centrifuged at 1600×g for 15 min at 4°C, and serum was separated and stored at −80°C until assayed. The serum activity of PON1 was measured as previously described (Mackness et al. 1997). The intra- and interassay coefficients of variance were <2.6 and <2.3%, respectively.

Statistical analysis

Quantitative data 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. BMD values were analyzed with or without adjustment for age by the least squares method in a general linear model (SAS, SAS Institute, Cary, NC, USA). Allele frequencies were estimated by the gene-counting method, and the chi-square test was used to identify significant departure from Hardy-Weinberg equilibrium. Haplotype analysis was performed with SAS/Genetics (SAS Institute). Correlation between the serum activity of PON1 and BMD was determined by simple regression analysis. Unless indicated otherwise, a P value of < 0.05 was considered statistically significant.

Results

The distribution of combined genotypes of three SNPs is shown in Table 1. Linkage disequilibrium was apparent between the 584A → G (Gln192Arg) and 172T → A (Leu55Met) SNPs of *PON1* [D', pairwise linkage disequilibrium coefficient ($D/D\max$), 0.4254; r, standardized linkage disequilibrium coefficient, 0.1673; P <0.0001], between the *PON1* 584A → G and *PON2* 959G → C (Cys311Ser) SNPs (D', 0.2112; r, 0.1470; P <0.0001), and between the *PON1* 172A → T and *PON2* 959G → C SNPs (D', 0.2491; r, 0.1421; P <0.0001).

Characteristics of study subjects according to genotypes for the 584A → G SNP or 172T → A SNP of *PON1* or for the 959G → C SNP of *PON2* are shown in Table 2. The distributions of genotypes were in Hardy-Weinberg equilibrium for premenopausal or postmenopausal women or for men in three SNPs. There was no difference in age or body mass index for premenopausal or postmenopausal women or for men among genotypes of each SNP.

The relation of the 584A → G SNP of *PON1* to BMD was examined (Table 3). To examine the possible influence of menopause on the relation between genotype and BMD, we analyzed BMD and other characteristics for premenopausal and postmenopausal women independently. Given the multiple comparisons of genotypes with BMD, we considered a P value of < 0.01 to be significant for such associations. For premenopausal women, BMD was not associated with 584A → G genotype with or without adjustment for age. In contrast, for postmenopausal women, BMD for the femoral neck was significantly lower in those with the *GG* genotype than in those with the *GA* or *AA* genotype. After adjustment for age, BMD for the femoral neck was also significantly lower in postmenopausal women with the *GG* genotype than in those with the *GA* or *AA* genotype or the *GA* genotype. The difference in adjusted BMD between the *GG* genotype and the *GA* or *AA* genotype (expressed as a percentage of the corresponding larger value) in postmenopausal women was

Table 1 Distribution of combined genotypes of polymorphisms of *PON1* and *PON2* among study subjects (n=2,196). Data are numbers of subjects (%)

PON1 584A → G	*PON1* 172T → A			*PON2* 959G → C
	TT	*TA*	*AA*	
GG	811 (36.93)	0 (0)	0 (0)	*CC*
	141 (6.42)	0 (0)	0 (0)	*CG*
	13 (0.59)	0 (0)	0 (0)	*GG*
GA	470 (21.40)	59 (2.69)	0 (0)	*CC*
	292 (13.30)	133 (6.06)	1 (0.05)	*CG*
	26 (1.18)	15 (0.68)	1 (0.05)	*GG*
AA	64 (2.91)	18 (0.82)	2 (0.09)	*CC*
	57 (2.60)	36 (1.64)	8 (0.36)	*CG*
	19 (0.87)	24 (1.09)	6 (0.27)	*GG*

Table 2 Characteristics of study subjects according to the 584A → G (Gln192Arg) or 172T → A (Leu55Met) genotype of *PON1* or the 959G → C (Cys311Ser) genotype of *PON2*. Data are means ± SE. *BMI* body mass index

Characteristic	584A → G (Gln192Arg)			172T → A (Leu55Met)			959G → C (Cys311Ser)		
	GG	*GA*	*AA*	*TT*	*TA*	*AA*	*CC*	*CG*	*GG*
Premenopausal women									
Number (%)	107 (38.6)	136 (49.1)	34 (12.3)	239 (86.0)	35 (12.6)	4 (1.4)	173 (62.2)	92 (33.1)	13 (4.7)
Age (years)	46.1 ± 0.4	46.2 ± 0.4	46.6 ± 0.8	46.0 ± 0.3	47.1 ± 0.8	48.3 ± 2.3	46.2 ± 0.4	46.2 ± 0.5	45.7 ± 1.3
BMI (kg/m^2)	22.9 ± 0.3	22.7 ± 0.3	22.7 ± 0.6	22.8 ± 0.2	22.5 ± 0.5	22.2 ± 1.6	22.9 ± 0.2	22.6 ± 0.3	22.8 ± 0.9
Postmenopausal women									
Number (%)	369 (45.6)	353 (43.6)	88 (10.9)	702 (86.2)	107 (13.1)	5 (0.6)	551 (67.5)	227 (27.8)	38 (4.7)
Age (years)	64.1 ± 0.4	63.8 ± 0.5	64.1 ± 0.9	64.1 ± 0.3	63.4 ± 0.8	63.4 ± 3.8	64.1 ± 0.4	63.5 ± 0.6	63.9 ± 1.4
BMI (kg/m^2)	22.8 ± 0.2	23.0 ± 0.2	23.5 ± 0.4	23.0 ± 0.1	22.7 ± 0.3	23.9 ± 1.5	22.9 ± 0.1	23.2 ± 0.2	23.3 ± 0.5
Men									
Number (%)	493 (44.3)	504 (45.3)	115 (10.3)	965 (86.3)	144 (12.9)	9 (0.8)	720 (64.0)	351 (31.2)	54 (4.8)
Age (years)	59.2 ± 0.5	59.2 ± 0.5	58.7 ± 1.0	59.0 ± 0.4	60.0 ± 0.9	59.4 ± 3.6	59.1 ± 0.4	59.2 ± 0.6	59.2 ± 1.5
BMI (kg/m^2)	22.9 ± 0.2	23.1 ± 0.2	22.6 ± 0.3	23.0 ± 0.1	22.8 ± 0.2	24.0 ± 1.2	23.1 ± 0.1	22.7 ± 0.2	23.0 ± 0.4

3.1% for the femoral neck. For men, BMD for the lumbar spine or femoral neck did not differ among 584A → G genotypes with or without adjustment for age (data not shown).

The relation between the 172T → A SNP of *PON1* and BMD is shown in Table 4. For premenopausal women, BMD was not associated with 172T → A genotype with or without adjustment for age. For postmenopausal women, BMD for the lumbar spine or femoral neck was significantly lower in those with the *TT* genotype than in those with the *TA* or *AA* genotype or the *TA* genotype, with or without adjustment for age. The differences in adjusted BMD between the *TT* genotype and the *TA* or *AA* genotype in postmenopausal women were 5.3% for the lumbar spine and 4.0% for the femoral neck. For men, BMD did not differ among 172T → A genotypes with or without adjustment for age (data not shown).

The relation of the 959G → C SNP of *PON2* with BMD is shown in Table 5. BMD did not differ among 959G → C genotypes for premenopausal women with or without adjustment for age. For postmenopausal women, BMD for the femoral neck was significantly lower in those with the *CC* genotype than in those with the *CG* or *GG* genotype with or without adjustment for age. The difference in adjusted BMD between the *CC* genotype and the *CG* or *GG* genotype in postmenopausal women was 2.9% for the femoral neck. BMD did not differ significantly among 959G → C genotypes in men with or without adjustment for age (data not shown).

We examined the relation of haplotypes of the 584A → G, 172T → A, and 959G → C SNPs to BMD (Table 6). Given the small number of subjects with the *GAC* (one woman and no men) and *GAG* (no women and one man) haplotypes, these groups were excluded from the analysis. For postmenopausal women, BMD

Table 3 Bone mineral density (BMD) of women (n = 1,087) according to the 584A → G (Gln192Arg) genotype of *PON1*. Data are means ± SE

Characteristic	Premenopausal (n = 277)				Postmenopausal (n = 810)			
	GG	*GA*	*AA*	*GA* + *AA*	*GG*	*GA*	*AA*	*GA* + *AA*
L2–L4	1.035 ± 0.012	1.026 ± 0.011	0.988 ± 0.021	1.018 ± 0.010	0.797 ± 0.008†	0.816 ± 0.008	0.830 ± 0.016	0.819 ± 0.007
Adjusted L2–L4[a]	1.034 ± 0.012	1.026 ± 0.010	0.990 ± 0.021	1.019 ± 0.009	0.798 ± 0.007†	0.814 ± 0.007	0.831 ± 0.015	0.818 ± 0.007
Femoral neck	0.777 ± 0.010	0.776 ± 0.008	0.745 ± 0.017	0.770 ± 0.008	0.632 ± 0.005‡‖	0.655 ± 0.006	0.649 ± 0.011	0.654 ± 0.005
Adjusted femoral neck[a]	0.776 ± 0.009	0.776 ± 0.008	0.747 ± 0.016	0.770 ± 0.007	0.633 ± 0.005§††	0.654 ± 0.005	0.650 ± 0.009	0.653 ± 0.004

[a]BMD with adjustment for age
†P < 0.05
‡P < 0.005
§P < 0.001 versus *GA* + *AA*
‖P < 0.01
††P < 0.005 versus *GA*

Table 4 Bone mineral density (BMD) of women (n = 1,092) according to the 172T → A (Leu55Met) genotype of *PON1*. Data are means ± SE

Characteristic	Premenopausal (n = 278)				Postmenopausal (n = 814)			
	TT	*TA*	*AA*	*TA* + *AA*	*TT*	*TA*	*AA*	*TA* + *AA*
L2–L4	1.030 ± 0.008	0.996 ± 0.021	0.940 ± 0.062	0.990 ± 0.020	0.803 ± 0.006†§	0.852 ± 0.015	0.849 ± 0.068	0.852 ± 0.014
Adjusted L2–L4[a]	1.029 ± 0.008	1.002 ± 0.020	0.954 ± 0.060	0.997 ± 0.019	0.803 ± 0.005‡§	0.848 ± 0.013	0.845 ± 0.061	0.848 ± 0.013
Femoral neck	0.775 ± 0.006	0.754 ± 0.017	0.709 ± 0.050	0.749 ± 0.016	0.639 ± 0.004†‖	0.672 ± 0.010	0.628 ± 0.047	0.670 ± 0.010
Adjusted femoral neck[a]	0.774 ± 0.006	0.758 ± 0.016	0.720 ± 0.048	0.755 ± 0.015	0.640 ± 0.003†§	0.669 ± 0.009	0.625 ± 0.039	0.667 ± 0.008

[a]BMD with adjustment for age
†P < 0.005
‡P < 0.001 versus *TA* + *AA*
§P < 0.005
‖P < 0.01 versus *TA*

Table 5 Bone mineral density (BMD) of women (n = 1,094) according to the 959G → C (Cys311Ser) genotype of *PON2*. Data are means ± SE

Characteristic	Premenopausal (n = 278)				Postmenopausal (n = 816)			
	CC	*CG*	*GG*	*CG* + *GG*	*CC*	*CG*	*GG*	*CG* + *GG*
L2–L4	1.028 ± 0.009	1.024 ± 0.013	0.983 ± 0.034	1.019 ± 0.012	0.803 ± 0.006	0.825 ± 0.010	0.814 ± 0.025	0.823 ± 0.009
Adjusted L2–L4[a]	1.028 ± 0.009	1.024 ± 0.013	0.979 ± 0.033	1.019 ± 0.012	0.804 ± 0.006	0.822 ± 0.009	0.814 ± 0.022	0.820 ± 0.008
Femoral neck	0.769 ± 0.008	0.778 ± 0.010	0.756 ± 0.028	0.775 ± 0.010	0.637 ± 0.004†‡	0.660 ± 0.007	0.655 ± 0.017	0.659 ± 0.006
Adjusted femoral neck[a]	0.770 ± 0.007	0.778 ± 0.010	0.753 ± 0.027	0.775 ± 0.009	0.638 ± 0.004†‡	0.657 ± 0.006	0.655 ± 0.014	0.657 ± 0.005

[a]BMD with adjustment for age
†P < 0.005 versus *CG* + *GG*
‡P < 0.05 versus *CG*

Table 6 Bone mineral density (BMD) of premenopausal or postmenopausal women or of men according to haplotypes of three polymorphisms. Data are means ± SE

BMD	Haplotype [584A → G (*PON1*), 172T → A (*PON1*), 959G → C (*PON2*)]					
	GTC	*GTG*	*ATC*	*ATG*	*AAC*	*AAG*
Premenopausal women						
No. of chromosomes (%)	314 (57.40)	31 (5.67)	106 (19.38)	55 (10.05)	9 (1.65)	32 (5.85)
L2–L4	1.034 ± 0.007	1.012 ± 0.022	1.014 ± 0.012	1.033 ± 0.017	0.999 ± 0.041	0.986 ± 0.022
Femoral neck	0.776 ± 0.006	0.783 ± 0.018	0.766 ± 0.010	0.781 ± 0.013	0.734 ± 0.033	0.751 ± 0.018
Postmenopausal women						
No. of chromosomes (%)	1016 (63.18)	66 (4.10)	259 (16.11)	150 (9.33)	33 (2.05)	84 (5.22)
L2–L4	0.802 ± 0.005	0.832 ± 0.019	0.817 ± 0.009	0.802 ± 0.012	0.869 ± 0.026	0.845 ± 0.017
Femoral neck	0.638 ± 0.003*	0.662 ± 0.013	0.644 ± 0.007	0.658 ± 0.009	0.700 ± 0.018	0.656 ± 0.011
Men						
No. of chromosomes (%)	1366 (62.03)	110 (5.00)	339 (15.40)	227 (10.31)	46 (2.09)	113 (5.13)
L2–L4	0.979 ± 0.004	0.970 ± 0.015	0.989 ± 0.009	0.988 ± 0.011	0.993 ± 0.024	0.984 ± 0.015
Femoral neck	0.750 ± 0.003	0.747 ± 0.011	0.757 ± 0.006	0.756 ± 0.008	0.765 ± 0.017	0.751 ± 0.011

*$P < 0.01$ versus *AAC*

Table 7 Serum activity (nmol min^{-1} ml^{-1}) of PON1 in study subjects according to the 584A → G (Gln192Arg) and 172T → A (Leu55Met) genotypes of *PON1* and the 959G → C (Cys311Ser) genotype of *PON2*. Data are means ± SE

	Total subjects	Women	Men
584A → G (Gln192Arg) genotype			
GG	503.7 ± 3.0 (n = 969)	516.5 ± 4.1 (n = 476)	491.2 ± 4.2 (n = 493)
GA	325.6 ± 2.9 (n = 993)*	333.5 ± 4.0 (n = 489)*	317.9 ± 4.1 (n = 504)*
AA	139.3 ± 6.1 (n = 237)*†	145.7 ± 8.3 (n = 122)*†	132.2 ± 8.9 (n = 115)*†
172T → A (Leu55Met) genotype			
TT	407.2 ± 3.3 (n = 1906)	413.9 ± 4.7 (n = 941)	400.5 ± 4.5 (n = 965)
TA	256.5 ± 8.4 (n = 286)‡	268.7 ± 12.2 (n = 142)‡	244.5 ± 11.5 (n = 144)‡
AA	101.9 ± 32.9 (n = 18)‡§	111.5 ± 49.1 (n = 9)‡‖	93.3 ± 43.9 (n = 9)‡‖
959G → C (Cys311Ser) genotype			
CC	408.9 ± 3.9 (n = 1444)	415.6 ± 5.6 (n = 724)	402.0 ± 5.5 (n = 720)
CG	349.1 ± 5.8 (n = 670)††	361.7 ± 8.4 (n = 319)††	337.4 ± 7.9 (n = 351)††
GG	285.2 ± 15.1 (n = 105)††#	267.3 ± 21.9 (n = 51)††#	302.4 ± 20.7 (n = 54)††

*P = 0.0001 versus *GG*
†P = 0.0001 versus *GA*
‡P = 0.0001 versus *TT*
§P = 0.0001 versus *TA*
‖P < 0.005 versus *TA*
††P = 0.0001 versus *CC*
#P < 0.0005 versus *CG*

for the femoral neck was significantly lower in those with the *GTC* haplotype than in those with the *AAC* haplotype. For premenopausal women or for men, BMD did not differ among haplotypes.

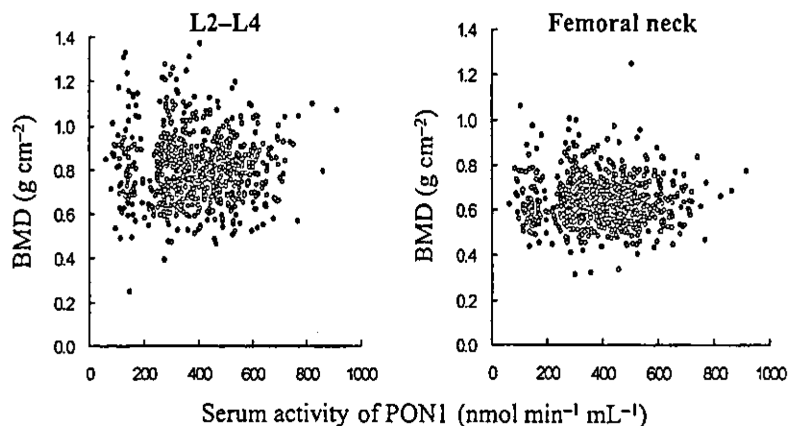
We examined the relation of three SNPs with the serum activity of PON1 (Table 7). There was a significant association between the serum activity of PON1 and 584A → G, 172T → A, and 959G → C genotypes both for women and for men. With regard to the 584A → G genotype, the activity of PON1 was higher in individuals with the *GG* genotype than in those with the *GA* genotype or those with the *AA* genotype; the activity was also higher in those with the *GA* genotype than in those with the *AA* genotype. For the 172T → A genotype, the activity of PON1 was higher in individuals with the *TT* genotype than in those with the *TA* or those with the *AA* genotypes; the activity was also higher in those with the *TA* genotype than in those with the *AA* genotype. With respect to the 959G → C genotype, the serum activity of PON1 was higher in individuals with the *CC* genotype than in those with the *CG* or those with the *GG* genotypes; for total subjects and for women, the activity was also higher in those with the *CG* genotype than in those with the *GG* genotype.

Finally, we examined the correlation between the serum activity of PON1 and BMD (Fig. 1). There was no significant relation of the serum activity of PON1 with BMD for the lumbar spine (r = 0.010, P = 0.844) or for the femoral neck (r = 0.014, P = 0.683) in postmenopausal women.

Discussion

We have shown that the 584A → G (Gln192Arg) and 172T → A (Leu55Met) SNPs of *PON1* and the 959G → C (Cys311Ser) SNP of *PON2* are associated with BMD for the lumbar spine or femoral neck in postmenopausal Japanese women, and that the 584*GG*, 172*TT*, and 959*CC* genotypes represent risk factors for

Fig. 1 Correlation of serum activity of PON1 with bone mineral density (BMD) for the lumbar spine (L2–L4) (*left panel*) or femoral neck (*right panel*) in postmenopausal women ($n=816$)



reduced bone mass. Haplotype analysis revealed that the *GTC* haplotype exhibited the lowest BMD and the *AAC* haplotype the highest BMD in postmenopausal women. There was no significant association of polymorphisms of *PON1* or *PON2* with BMD in premenopausal women or in men.

The three SNPs examined in the present study have been previously associated with the activity of PON1 in serum or plasma. We measured the serum activity of PON1 in the present study population to confirm the effects of three SNPs on the activity. Humbert et al. (1993) determined that the 584*G* (192Arg) allele is associated with a higher activity of PON1 in plasma than is the 584*A* (192Gln) allele. Garin et al. (1997) showed that the plasma concentrations and activities of PON1 decreased according to the rank order of 172T → A (Leu55Met) genotypes *TT* > *TA* > *AA*. Our present results are consistent with these previous observations (Garin et al. 1997; Humbert et al. 1993).

Mackness et al. (2000) showed that the 959G → C (Cys311Ser) SNP of *PON2* also affects the serum activity of PON1; among individuals with type 2 diabetes, PON1 activity was highest in those with the 959*GG* genotype. In contrast, our results demonstrate that the *GG* genotype is associated with the lowest PON1 activity and that the *CC* genotype exhibits the highest activity for both women and men.

In the present study, the 584*GG*, 172*TT*, and 959*CC* genotypes, which exhibited the highest serum activity of PON1, were associated with reduced BMD in postmenopausal women. A high serum activity of PON1 may result in a reduced concentration of lipid peroxidation products and might therefore be expected to prevent bone loss (Basu et al. 2001; Garrett et al. 1990; Parhami et al. 1997; Parhami et al. 1999; Parhami et al. 2000). The association between genotypes and BMD in our study is thus opposite to that anticipated from such a mechanism. The molecular mechanisms that underlie the association of SNPs of *PON1* and *PON2* with BMD thus remain unclear. It is possible that the SNPs examined in our study are in linkage disequilibrium with polymorphisms of other nearby genes that are determinants of BMD. Indeed, the interleukin-6 gene (7q21) and calcitonin receptor gene (7q21.3), both of which have been associated with BMD (Ota et al. 1999; Ota et al. 2001; Taboulet et al. 1998), are located close to *PON1* and *PON2* (7q21-q22). However, our present results suggest that *PON1* and *PON2* are candidate loci for reduced bone mass in postmenopausal Japanese women.

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ORIGINAL ARTICLE

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Genetic deficiency of a mitochondrial aldehyde dehydrogenase increases serum lipid peroxides in community-dwelling females

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Abstract Mitochondrial aldehyde dehydrogenase 2 (ALDH2) plays a major role in acetaldehyde detoxification. The alcohol sensitivity is associated with a genetic deficiency of ALDH2. We and others have previously reported that such a deficiency influences the risk for late-onset Alzheimer's disease (LOAD), hypertension, and myocardial infarction. Then we tried to find phenotypes to which the *ALDH2* polymorphism contributes by conducting several evaluations including biochemical and functional analyses of various tissues in a community-dwelling population. Several serum proteins, lipids, and lipid peroxides (LPO) levels showed differences between the nondefective (*ALDH2*1/1*) and defective (*ALDH2*1/2* and *ALDH2*2/2*) ALDH2 individuals. However, alcohol-drinking behavior is known to affect these evaluations. Thus, we excluded the effects of alcohol-drinking behavior from the association with the ALDH2-deficient genotype through correction and found that the concentration of LPO was significantly lower in the nondefective ALDH2 females than the defective females. The effect of frequent alcohol-drinking behavior in males seems to override the phenotype of the high serum LPO level. These results indicate that the ALDH2 deficiency may enhance oxidative stress in vivo. Thus, these findings suggest that ALDH2 functions as a protector against oxidative stress and the decrease in protection may influence the onset of AD, hypertension, and myocardial infarction.

Keywords Aldehyde dehydrogenase 2 · Gene polymorphism · Lipid peroxides · Population-based study · Alzheimer's disease

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Introduction

Mitochondrial aldehyde dehydrogenase 2 (ALDH2) metabolizes acetaldehyde produced from ethanol into acetate and plays a major role in the oxidation of acetaldehyde in vivo (Bosron and Li 1986). A mutant allele, *ALDH2*2*, has a single point mutation (G → A) in exon 12 of the active *ALDH2*1* gene and is confined to Asians (Yoshida et al. 1984). The mutation results in a substitution of glutamic acid 487 to lysine (E487 K), acting in a dominant negative fashion (Crabb et al. 1989, Singh et al. 1989, Xiao et al. 1996). Individuals with the *ALDH2*2* allele exhibit the alcohol-flushing syndrome attributable to an elevated blood acetaldehyde level (Goedde et al. 1979, Crabb 1990). The *ALDH2*2* allele has been also reported to affect the metabolism of other aldehydes such as benzaldehyde, which is a metabolite of toluene (Kawamoto et al. 1994), and chloroacetaldehyde, which is generated during the metabolism of vinyl chloride (Farres et al. 1994, Yokoyama et al. 1996). In addition, ALDH2 deficiency was found to contribute to risks of hypertension (Takagi et al. 2001, Amamoto et al. 2002) and myocardial infarction (Takagi et al. 2002). However, the risks have been mainly argued through an association with alcohol consumption.

Recently, we have reported that ALDH2 deficiency is a risk factor for late-onset Alzheimer's disease (LOAD), synergistically acting with the $\epsilon 4$ allele of the apolipoprotein E gene (*APOE-*$\epsilon 4$) (Kamino et al. 2000). LOAD is a complex disease caused by multiple genetic and environmental factors: physiological, medical, nutritional,

and psychological. Oxidative stress and lipid peroxidation caused by reactive oxygen species (ROS) are reported to play an important role in the pathogenesis of neurodegenerative diseases. These diseases include Alzheimer's disease (AD) (Lovell et al. 1997, Mark et al. 1997), Parkinson's disease (Dexter et al. 1994), amyotrophic lateral sclerosis (Ferrante et al. 1997, Pedersen et al. 1998), and cerebral ischemia (for review, Chan 2001). A major source of ROS is mitochondrially derived superoxide anion radical, which gives rise to hydrogen peroxide. Hydrogen peroxide is often converted further to hydroxyl radical. Superoxide anion reacts with unsaturated fatty acids and induces membrane lipid peroxidation thereby generating reactive aldehydes, including malondialdehyde (MALD) and trans-4-hydroxy-2-nonenal (4-HNE). A strong electrophile, 4-HNE, has the ability to readily adduct cellular proteins and may damage the proteins by interacting with lysine, histidine, serine, and cysteine residues (Uchida and Stadtman 1992). Recently, we found that ALDH2-deficient transfectants exhibited increased vulnerability to treatment with 4-HNE (Ohsawa et al. 2003). The transfectants also had a decreased resistance to oxidative insult, caused by antimycin A, accompanied by an accumulation of proteins modified with 4-HNE. These findings suggest that mitochondrial ALDH2 functions as a protector against oxidative stress and its deficiency increases the damage from oxidative stress.

Geriatric diseases including LOAD are associated with many factors: genetic, lifestyle, physiological, medical, nutritional, and psychological. Thus, it is important to clarify the contributions of genetic factors and other basic background factors. In 1997, we started gene-related investigations into various geriatric diseases in the National Institute for Longevity Sciences, Longitudinal Study of Aging (NILS-LSA) (Shimokata et al. 2000). In this study, a molecular epidemiological analysis in the NILS-LSA revealed a higher concentration of lipid peroxides (LPO) in sera of ALDH2-deficient females than females carrying an active ALDH2. These results suggest that ALDH2 is involved in antioxidant defense and its deficiency enhances oxidative stress.

Subjects and methods

Molecular epidemiological study

The subjects were 2,259 participants in the NILS-LSA study. They were randomly selected community-dwelling males and females aged 40–79 years from Obu City and regions close to the NILS in Aichi Prefecture, Japan. The study protocol was approved by the Committee on the Ethics of Human Research of National Chubu Hospital and the NILS, and written informed consent was obtained from each subject. Their venous blood (7 ml) was collected into tubes containing EDTA (final 50 mM), and genomic DNA was isolated with an automated genomic DNA isolation system (model NA-1000; Kurabo, Osaka, Japan). The genotype of *ALDH2* was determined by the mismatched polymerase chain reaction (PCR)-RFLP method reported previously (Shimokata et al. 2000). In brief, 5 ng of DNA was amplified in 15 μl of PCR mixture with the primers 5'-TTACAGGGTCAACTGCTATG-3' and 5'-CCACACTCACA-3'. The amplified 131-bp DNA fragment including exon 12 of the *ALDH2* gene was digested with *Ear*I and separated by agarose gel electrophoresis. The *ALDH2*1* allele has 108 and 23 bp, and the mutant *ALDH2*2* allele 131 bp. Routine clinical evaluations included physical examination, blood pressure, blood chemistry including LDH, total cholesterol, triglyceride, HDL-cholesterol, LPO, complete blood cell count, and urine analysis. LPO was determined as thiobarbituric acid reactive substances.

Statistical analysis

Data are presented as means ± SD. LPO and other quantitative data were compared among ALDH2 genotypes by one-way analysis of variance and the Tukey-Kramer post hoc test. Alcohol drinking was defined as >5 g alcohol per day in Table 3, and alcohol consumption was assessed as a continuous (grams alcohol per day) variable in Tables 4 and 5. In Table 2, data were analyzed with an adjustment for alcohol consumption by the least squares method in a general linear model. In Table 5, data were analyzed by the general linear model with both *ALDH2* genotype and alcohol consumption used as an independent variable. A p value of 0.05 or less after correction by the number of comparisons was considered statistically significant.

Results

We first examined the distribution of the *ALDH2* genotype in the NILS-LSA study. The subjects numbered 2,259. They were community-dwelling males and females aged 40–79 years who were randomly selected from the area of the NILS. The genotype frequencies for *ALDH2*1/1*, *1/2* and *2/2* were tested in 1,137 males and 1122 females (Table 1). The overall frequencies of genotypes *1/1*, *1/2* and *2/2* were 51.1%, 40.1%, and 8.8%, respectively. There was no gender difference in the genotypic frequencies. However, the frequency of genotype *1/1* showed a trend in an increase (from 49.1% in the 40s group to 53.1% in the 70s group), depending upon age, despite no statistical significance. We tried to find a combination to show the significance. Then, only when females were divided into two groups (*ALDH2*1/1* and *1/2*) or (*2/2*) by age, the frequency of *ALDH2*2/2* was marginally, but significantly, lower in the older group (≥60 years) than the younger group (<60 years) ($p=0.03$ chi-square analysis, or $p=0.02$ by Fisher's exact test). Thus, the frequencies of these genotypes may not be constant throughout life. We examined the association of the *ALDH2*-deficient genotype with various evaluations in the NILS-LSA study. In addition to biochemical analyses of blood and urine, renal and liver functions, serum proteins and lipids, and a complete blood count, LPO and geriatric disease markers were also examined. Several serum proteins, lipids, and LPO levels showed differences between the nondefective (*ALDH2*1/1*) and the defective (*ALDH2*1/2* and *ALDH2*2/2*) ALDH2 individuals (Table 2). However, these biochemical evaluations are known to be affected by alcohol-drinking behavior (29). Indeed, subjects with the *ALDH2*1/1* genotype drank alcohol more frequently than those with *ALDH2*1/2* and *2/2* (Table 3).

Table 1 Genotypic frequencies for *ALDH2*

Subjects	Number	*ALDH2* genotype			
		1/1	*1/2*	*2/2*	p*
Male	1137	590 (51.9%)	449 (39.5%)	98 (8.6%)	
Female	1127	565 (50.1%)	461 (40.9%)	101 (9.0%)	0.71
Age					
40s	573	280 (48.9%)	241 (42.1%)	52 (9.1%)	
50s	561	285 (50.8%)	224 (39.9%)	52 (9.3%)	
60s	569	292 (51.3%)	222 (39.0%)	55 (9.7%)	
70s	561	298 (53.1%)	223 (39.8%)	40 (7.1%)	0.65
Total	2264	1155 (51.0%)	910 (40.2%)	199 (8.8%)	

*Comparison of genotype distributions by gender (Chi-square statistics)

Female	*ALDH2* genotype	
	1/1 and *1/2*	*2/2*
Age 40s and 50s	498 (89.25%)	60 (10.75%)
Age 60s and 70s	524 (92.91%)	40 (7.09%)

Chi-square statistics; $p=0.031$
Fisher's exact test (left); $p=0.020$

Table 2 Effects of *ALDH2* genotypes on serum lipid, lipid protein, and lipid peroxides

Gender	Male			Female		
ALDH2 genotypes Evaluations[b]	*1/1*	*1/2* & *2/2*	p[a]	*1/1*	*1/2* & *2/2*	p
TG	140.7±3.9[c]	126.7±4.1	0.014*	107.8±2.6	110.5±2.6	0.462
Total chol	212.4±1.4	212.0±1.4	0.838	226.8±1.5	227.3±1.5	0.819
HDL	58.84±0.61	55.91±0.63	0.001*	67.01±0.64	65.06±0.65	0.033*
LPO	3.118±0.029	3.072±0.030	0.267	2.815±0.030	2.946±0.030	0.002*
LDL	130.0±1.3	133.7±1.4	0.055	138.4±1.5	140.5±1.5	0.316
APO A1	145.5±1.1	138.2±1.2	<0.001*	157.5±1.1	153.8±1.1	0.021*
APO A2	38.69±0.24	37.11±0.25	<0.001*	38.13±0.23	37.45±0.23	0.042*
APO B	108.0±1.0	108.7±1.0	0.594	109.6±1.1	110.8±1.1	0.405
APO C2	4.778±0.055	4.490±0.057	<0.001*	4.590±0.053	4.510±0.053	0.283
APO C3	11.47±0.13	10.58±0.14	<0.001*	10.87±0.11	10.76±0.11	0.479
APO E	4.762±0.053	4.568±0.055	0.012*	5.010±0.050	4.999±0.051	0.886

*$p<0.05$
[a]Comparison by *ALDH2* genotypes in each gender (Tukey-Kramer)
[b]TG: Triglyceride (mg/dl), Total chol, total cholesterol (mg/dl); HDL, high density lipoprotein cholesterol (mg/dl); LPO, lipid peroxide (nmol/ml); LDL, low-density lipoprotein cholesterol (mg/dl); Apo, apolipoprotein (mg/dl)
[c]Concentrations are means ± SDs

Table 3 *ALDH2* genotypes and alcohol-drinking behavior

Subjects	Number	*ALDH2* genotype	Drinking[a]		
			Yes	No	Total
Male	1137	*1/1*	475 (87.3%)	69 (8.6%)	544
		1/2	234 (56.6%)	179 (43.3%)	413
		2/2	4 (4.3%)	89 (95.7%)	93
Female	1127	*1/1*	187 (36.3%)	328 (63.7%)	515
		1/2	62 (14.8%)	356 (85.2%)	418
		2/2	4 (4.3%)	90 (95.7%)	94

[a]Alcohol drinking was defined as > 5 g alcohol per day

Thus, we excluded the effects of alcohol-drinking behavior from the association of the *ALDH2*-deficienct genotype with the evaluation.Data were analyzed with an adjustment for alcohol consumption by the least squares method in the general linear model (Table 4), and we found that the concentration of LPO in females differed significantly by *ALDH2* genotype. The concentration was higher in females carrying at least one *ALDH2*2* allele (2.922 nmol/ml) than those carrying *ALDH2*1/1* (2.781 nmol/ml; $p=0.003$), indicating the possibility that oxidative stress increases in *ALDH2*-deficient individuals. To find why the significance was found only in the females, the general linear model was applied with both *ALDH2* genotype and alcohol consumption used as an independent variable. As a result, the concentration of LPO was significantly determined

Table 4 Effects of *ALDH2* genotypes on serum lipid, lipid protein, and lipid peroxides (LPO): Exclusion of effects of alcohol-drinking behavior

Gender	Male			Female		
ALDH2 genotypes Evaluations[b]	*1/1*	*1/2* & *2/2*	p^a	*1/1*	*1/2* & *2/2*	p
TG	138.0±4.3[c]	131.2±4.5	0.288	109.7±2.8	111.4±2.8	0.679
Total chol	212.9±1.5	211.4±1.6	0.492	227.2±1.5	226.0±1.6	0.566
HDL	57.71±0.65	56.69±0.67	0.291	66.48±0.66	64.68±0.67	0.057
LPO	3.054±0.031	3.075±0.032	0.640	2.781±0.033	2.922±0.033	0.003*
LDL	131.8±1.5	132.2±1.5	0.835	139.3±1.5	139.5±1.6	0.921
APO A1	143.0±1.2	140.9±1.3	0.250	157.2±1.2	154.3±1.2	0.076
APO A2	38.12±0.25	37.92±0.26	0.586	38.20±0.25	37.61±0.25	0.101
APO B	108.6±1.1	108.1±1.1	0.750	109.9±1.1	110.4±1.1	0.774
APO C2	4.741±0.060	4.596±0.062	0.105	4.654±0.056	4.547±0.056	0.183
APO C3	11.22±0.14	10.94±0.15	0.173	10.98±0.12	10.80±0.12	0.270
APO E	4.727±0.058	4.627±0.060	0.253	5.031±0.053	5.000±0.054	0.693

*p <0.05
[a]Comparison by *ALDH* genotypes in each gender (Tukey-Kramer)
[b]TG, Triglyceride (mg/dl); Total chol, total cholesterol (mg/dl); HDL, high density lipoprotein cholesterol (mg/dl); LPO, lipid peroxide (nmol/ml); LDL, low-density lipoprotein cholesterol (mg/dl); Apo, apolipoprotein (mg/dl)
[c]Concentrations are means ± SDs

Table 5 Determinants of lipid peroxide[a]

	Determinants[b]	p^c
Male	*ALDH2*	0.640
	Alcohol	0.001
Female	*ALDH2*	0.003
	Alcohol	0.247

[a]Data were analyzed by the general linear model with both *ALDH2* genotype and alcohol consumption as an independent variable
[b]The *ALDH2* genotypes were categorized into two groups, *1/1* or *1/2* + *2/2*. "Alcohol" indicates alcohol consumption, which was assessed as a continuous (grams alcohol per day) variable
[c]*F*-test

by alcohol consumption (p=0.001) in males while by ALDH2 genotype (p=0.003) in females (Table 5). Thus, a concentration of LPO in males was influenced by alcohol-drinking behavior.

Discussion

ALDH2 plays a major role in the oxidation of acetaldehyde in vivo. Its low *Km* facilitates the rapid clearance of acetaldehyde following the administration of alcohol, and a deficiency of ALDH2 results in an ethanol-related sensitive response attributable to an elevated blood acetaldehyde level. Several reports have suggested that an increase in the acetaldehyde concentration is a risk for diabetes (Suzuki et al. 1996a, 1996b), cancer (Yokoyama et al. 1998), and hypertension (Itoh et al. 1997) and is associated with *ALDH2*2*. Thus, the risk for geriatric diseases, including myocardial infarction, has been mainly argued through an association with alcohol consumption.

Instead, this study has revealed that ALDH2 could contribute to the pathogenesis of various geriatric diseases by an alternative pathway. Since the LPO concentration in sera of females carrying *ALDH2*2* was higher, even after correcting for alcohol-drinking behavior, the increase of LPO in ALDH-deficient individuals would not be due to drinking. This suggests that ALDH2 might contribute to the elimination of not only acetaldehyde from ethanol but also aldehyde derivatives produced by oxidative stress. We found no significant difference in males in the concentration of serum LPO among *ALDH2* genotypes. However, an analysis in the general linear model with both the *ALDH2* genotype and alcohol consumption used as an independent variable indicates that a determinant of the concentration of LPO is alcohol consumption in males. This finding strongly suggesting that frequent alcohol-drinking behavior in males overrides the phenotype of the high serum LPO level. Alternatively, it cannot exclude the possibility that some hormonal regulation contributes to the peroxidation.

Our previous case-control study has revealed that ALDH2 deficiency is a risk factor for LOAD in a Japanese population, synergistically acting with APOE-ϵ4 (Kamino et al. 2000). Oxidative stress has been primarily implicated in mechanisms of AD brain degeneration (Markesbery and Carney 1999, Praticò and Delanty 2000). A mouse model of AD amyloidosis showed evidence of a systemic increase in urine, plasma, and brain LPO compared with wild-type mice (Praticò et al. 2001). The increase preceded the onset of amyloid deposition. Oxidative damage to the central nervous system predominantly manifests as LPO because of the high level of polyunsaturated fatty acids that are particularly susceptible to oxidation. Thus, the higher LPO concentration in sera of females carrying *ALDH2*2* might be reflected by the higher frequency of LOAD in females.

Mechanisms underlying the increase of LPO in ALDH2-deficient individuals should be further investigated. However, one possible explanation is as follows: The enhanced accumulation of toxic acetaldehyde or

aldehyde derivatives including 4-HNE in the ALDH2-deficient cells induces cell death, and cellular damage induced by the accumulated aldehyde derivatives further enhances oxidative stress in vivo. Several studies have revealed that 4-HNE, an aldehyde derivative of membrane lipid peroxidation, is a key mediator of neuronal apoptosis induced by oxidative stress and that a protein modification by 4-HNE increases in the AD brain (Montine et al. 1997, Sayre et al. 1997).

The metabolism of 4-HNE in hepatocytes has been reported to be dependent on three enzymatic pathways: oxidation with ALDH, reduction with alcohol dehydrogenase, and conjugation with glutathione (Hartley et al. 1995). Recently, we found that ALDH2 deficiency in PC12 cells increased cell death after treatment with cytotoxic 4-HNE (Ohsawa et al. 2003). Furthermore, after treatment with antimycin A, the ALDH2 deficiency resulted in an enhancement of both 4-HNE accumulation and cell death. These results strongly support our explanation described above.

Finally, our results suggest possible roles of ALDH2 deficiency in the increase in LPO, which is produced in response to oxidative stress. Even on excluding the effects of alcohol-drinking behavior from the association with the *ALDH2*-deficienct genotype using the correction, the concentration of LPO in females significantly differed by *ALDH2* genotype. However, the effect of frequent alcohol-drinking behavior in males seems to override the phenotype of the high serum LPO level. LPO and its derivative, 4-HNE, are involved in the pathogenesis of several geriatric diseases including AD, and ALDH2 may detoxify them. Thus, the metabolism of toxic aldehydes, including 4-HNE, could be a preventive and therapeutic target in geriatric diseases.

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