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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).

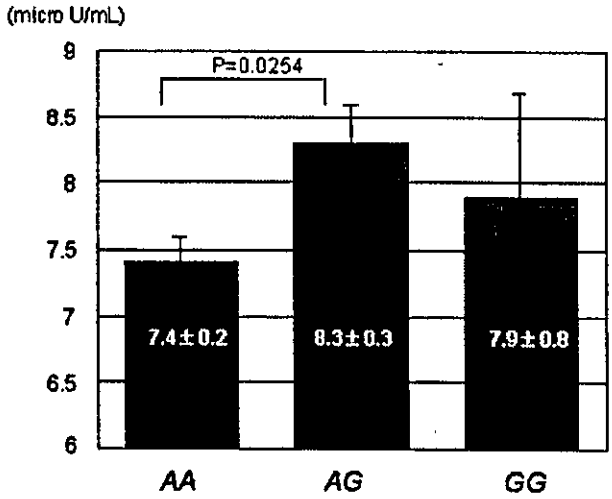

Figure 1 Comparison of fasting insulin levels among A→G genotypes in middle-aged women.

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

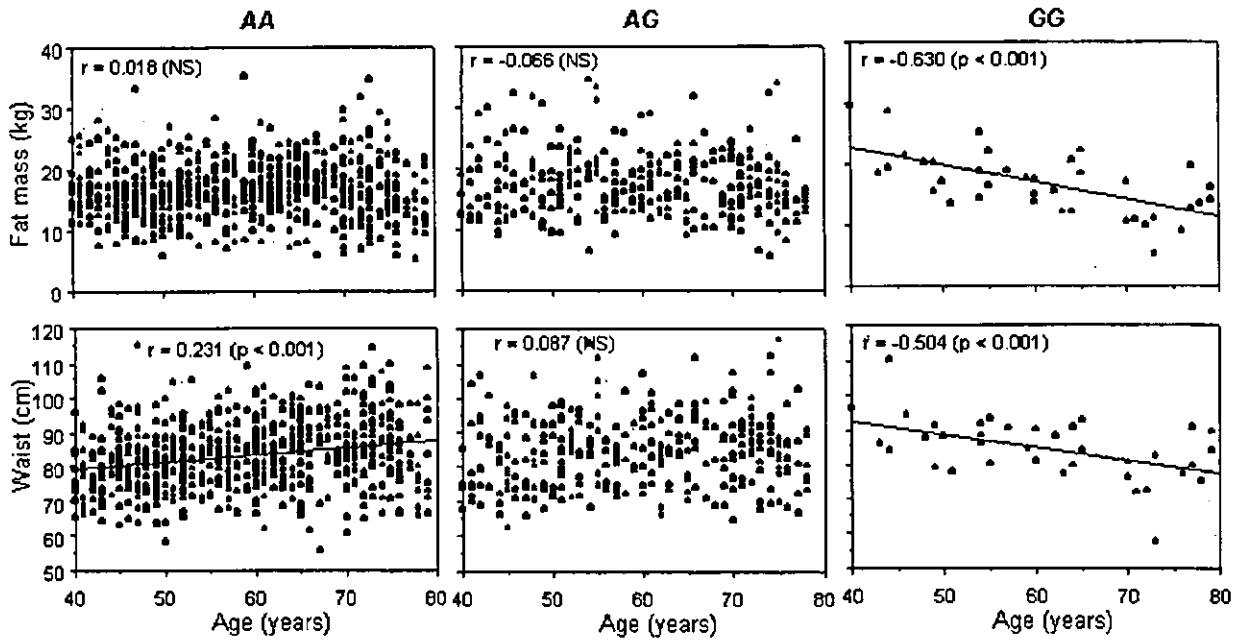

Figure 2 Relations of age with fat mass and waist by each A→G genotype in women.

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 ARTICLE

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Association of a polymorphism of the dopamine receptor D4 gene with bone mineral density in Japanese men

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Abstract A –521C → T polymorphism in the promoter of the dopamine receptor D4 gene (*DRD4*) has been associated with novelty-seeking behavior in Japanese. Given that the dopaminergic system might also play an important role in bone metabolism, the relation of the –521C → T polymorphism of *DRD4* to bone mineral density (BMD) was examined in 2,228 Japanese subjects (1,123 men; 1,105 women) who were randomly recruited to a population-based, prospective cohort study. 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. Genotype was determined with a fluorescence-based allele-specific DNA primer assay system. For men, BMD for the distal radius, total body, lumbar spine, trochanter, or Ward's triangle was lower in individuals with the *CC* genotype than in the combined group of *TT* and *TC* genotypes or in those with the *TT* genotype or with the *TC* genotype. The urinary concentration of deoxypyridinoline was slightly, but significantly, greater in men with the *CC* genotype than in those with the *TT* or *TC* genotypes or with the *TT* genotype. For women, there were no differences in BMD among –521C → T genotypes. These results implicate *DRD4* as a candidate locus for reduced BMD in Japanese men.

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Introduction

The dopaminergic system participates in various physiological processes that affect cardiovascular-renal function, including control of blood pressure and maintenance of electrolyte balance, as well as in reproduction and regulation of cell calcium homeostasis (Gilbert 1993). Altered dopaminergic activity may result in changes not only in renal sodium balance, but also in the renal response to vitamin D, calcium, and parathyroid hormone. Such altered interactions among vitamin D, calcium, and sodium in the kidney have implications for bone metabolism (Gilbert 1993). Dopamine is also essential for ovarian function, and estrogens and androgens in turn increase dopamine turnover. An altered dopaminergic status may thus affect actions of these sex hormones that are associated with qualitative and quantitative attributes of bone (Gilbert 1993).

The dopamine receptor D4 is expressed in limbic areas of the brain that contribute to cognition and emotion (Van Tol et al. 1991) and mediates exploratory behavior in experimental animals (Fink and Smith 1980). Sequence variability within the dopamine receptor D4 gene (*DRD4*) has been implicated in the personality trait of novelty seeking, which is related to extroversion (Benjamin et al. 1996; Ebstein et al. 1996). A C-to-T single nucleotide polymorphism (SNP) at position –521 of *DRD4* relative to the translation start site has been associated with novelty-seeking behavior in independent studies of Japanese (Okuyama et al. 2000) and Hungarians (Ronai et al. 2001). With the use of a transient expression system, Okuyama et al. (2000) showed that the transcriptional activity of the *–521T* allele of *DRD4* was reduced by ~40% compared with that of the *–521C* allele in a human retinoblastoma cell

line, suggesting functional relevance of this SNP in dopaminergic neurotransmission.

We have now examined whether the −521C → T SNP in the promoter of *DRD4* is associated with bone mineral density (BMD) in Japanese men or women in a population-based study.

Materials 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; Yamada et al. 2003). 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 present study represents a cross-sectional analysis of the NILS-LSA population. 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 that affect bone metabolism, such as estrogen, progesterone, glucocorticoids, and bisphosphonates, were also excluded. We examined the relation of BMD at various sites to the −521C → T SNP of *DRD4* in 2,228 participants (1,123 men; 1,105 women). 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 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, USA). 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 *DRD4* genotype

Genotypes were determined with a fluorescence-based allele-specific DNA primer assay system (Toyobo Gene Analysis, Tsuruga, Japan) (Yamada et al. 2002). The polymorphic region of *DRD4* was amplified by the polymerase chain reaction with allele-specific sense primers labeled at the 5' end with either fluorescein isothiocyanate (5'- GAGCGGGCGTGGAGGX*C*G-3') or Texas red (5'-GAGCGGGCGTGGAGGX*T*C-3') and with an antisense primer labeled at the 5' end with biotin (5'- GACTCGCCTCGACCTCGTG-3'). The reaction mixtures (25 μl) contained 20 ng of DNA, 5 pmol of each primer, 0.2 mmol/l of each deoxynucleoside triphosphate, 2 mmol/l $MgCl_2$, and 1 U of rTaq DNA polymerase (Toyobo, Osaka, Japan) in polymerase buffer. The amplification protocol comprised initial denaturation at 95°C for 5 min; 35 cycles of denaturation at 95°C for 30 s, annealing at 65°C for 30 s, and extension at 72°C for 30 s; and a final extension at 72°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.

Measurement of biochemical markers of bone turnover

Venous blood and urine samples were collected in the early morning after the subjects had fasted overnight. Blood samples were centrifuged at 1,600×g for 15 min at 4°C, and the serum fraction was separated and stored at −80°C until assayed. The serum concentration of osteocalcin was measured with an immunoradiometric assay kit (Mitsubishi Chemical, Tokyo, Japan). The activity of bone-specific alkaline phosphatase (ALP) in serum was measured with an enzyme immunoassay kit (Metra Biosystems, Mountain View, CA, USA). Urine samples were collected in plain tubes and stored at −80°C. Urinary deoxypyridinoline was measured with an enzyme immunoassay kit (Metra Biosystems); the values were corrected for urinary creatinine and expressed as picomoles per micromole of creatinine. Urinary creatinine was enzymatically measured with a creatinine test kit (Wako Chemical, Osaka, Japan).

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 adjustment for age and body mass index (BMI) by the least squares method in a general linear model. Allele frequencies were estimated by the gene-counting method, and the chi-square test was used to identify significant departure from Hardy-Weinberg equilibrium. A P value of < 0.05 was considered statistically significant.

Results

Characteristics of male subjects are shown in Table 1. The distribution of −521C → T genotypes of *DRD4* was in Hardy-Weinberg equilibrium for men. Age, BMI, and the prevalence of smoking did not differ among −521C → T genotypes. Height and body weight were greater in men with the *TC* genotype or in the combined group of *TT* and *TC* genotypes than in men with the *CC* genotype. After adjustment for age and BMI, BMD for the distal radius (D50), total body, lumbar spine, trochanter, or Ward's triangle was significantly lower in men with the *CC* genotype than in the combined group of *TT* and *TC* genotypes or in those with the *TT* genotype or with the *TC* genotype (Table 2). The differences in adjusted BMD between the *CC* genotype and the combined *TT*-*TC* group (expressed as a percentage of the corresponding larger value) were 4.8% (D50), 1.6% (total body), 3.0% (lumbar spine), 3.6% (trochanter), and 4.3% (Ward's triangle).

Table 1 Characteristics of men ($n = 1,123$) according to the −521C → T genotype of *DRD4*

Characteristic	*TT*	*TC*	*TT + TC*	*CC*
Number (%)	393 (35.0)	553 (49.2)	946 (84.2)	177 (15.8)
Age (years)	58.8 ± 0.6	59.1 ± 0.5	59.0 ± 0.4	60.3 ± 0.8
Height (cm)	164.6 ± 0.3	164.8 ± 0.3*	164.7 ± 0.2*	163.4 ± 0.5
Body weight (kg)	62.2 ± 0.5	62.7 ± 0.4*	62.5 ± 0.3†	60.4 ± 0.7
Body mass index (kg/m2)	22.9 ± 0.1	23.0 ± 0.1	23.0 ± 0.1	22.6 ± 0.2
Smoking (%)	38.9	37.3	37.9	40.0

Data are means ± SE
*P < 0.05
†P < 0.01 versus *CC*

Table 2 Bone mineral density (BMD) of men according to the −521C → T genotype of *DRD4*. *pQCT* peripheral quantitative computed tomography

Site	*TT*	*TC*	*TT + TC*	*CC*
BMD values measured by pQCT (mg/cm3)				
D50	266.4 ± 3.3	269.8 ± 2.8*	268.4 ± 2.1*	255.5 ± 4.9
D100	540.1 ± 4.7	543.5 ± 3.9	542.1 ± 3.0	531.6 ± 6.8
P100	1187.8 ± 7.2	1183.9 ± 6.0	1185.5 ± 4.6	1182.3 ± 10.6
BMD values measured by DXA (g/cm2)				
Total body	1.090 ± 0.005	1.089 ± 0.004	1.090 ± 0.003*	1.073 ± 0.007
L2–L4	0.988 ± 0.008	0.987 ± 0.006	0.987 ± 0.005*	0.957 ± 0.011
Femoral neck	0.757 ± 0.005	0.754 ± 0.004	0.755 ± 0.003	0.741 ± 0.008
Trochanter	0.674 ± 0.005†	0.670 ± 0.004*	0.672 ± 0.003‡	0.648 ± 0.007
Ward's triangle	0.558 ± 0.006*	0.554 ± 0.005	0.556 ± 0.004*	0.532 ± 0.009

Data are means ± SE
BMD values are adjusted for age and body mass index
*P < 0.05
†P < 0.01
‡P < 0.005 versus *CC*

Table 3 Characteristics of women ($n = 1,105$) according to the −521C → T genotype of *DRD4*

Characteristic	*TT*	*TC*	*TT + TC*	*CC*
Number (%)	400 (36.2)	508 (46.0)	908 (82.2)	197 (17.8)
Age (years)	59.4 ± 0.5	59.9 ± 0.5*	59.7 ± 0.4*	57.6 ± 0.8
Height (cm)	151.1 ± 0.3	151.0 ± 0.3	151.0 ± 0.2*	152.1 ± 0.4
Body weight (kg)	52.5 ± 0.4	52.4 ± 0.4	52.5 ± 0.3	52.2 ± 0.6
Body mass index (kg/m2)	23.0 ± 0.2	23.0 ± 0.1	23.0 ± 0.1	22.6 ± 0.2
Smoking (%)	8.3	5.3	6.6	9.6

Data are means ± SE
*P < 0.05 versus *CC*

Table 4 Bone mineral density (BMD) of women according to the −521C → T genotype of *DRD4*. *pQCT* peripheral quantitative computed tomography

Site	*TT*	*TC*	*TT + TC*	*CC*
BMD values measured by pQCT (mg/cm3)				
D50	183.2 ± 3.1	186.3 ± 2.7	185.0 ± 2.1	183.9 ± 4.5
D100	485.0 ± 4.5	484.3 ± 4.0	484.6 ± 3.0	486.2 ± 6.5
P100	1157.9 ± 7.3	1145.7 ± 6.4	1151.1 ± 4.8	1153.4 ± 10.5
BMD values measured by DXA (g/cm2)				
Total body	0.965 ± 0.004	0.965 ± 0.004	0.965 ± 0.003	0.964 ± 0.006
L2–L4	0.864 ± 0.006	0.867 ± 0.006	0.865 ± 0.004	0.864 ± 0.009
Femoral neck	0.676 ± 0.004	0.678 ± 0.004	0.677 ± 0.003	0.678 ± 0.006
Trochanter	0.569 ± 0.004	0.571 ± 0.004	0.570 ± 0.003	0.572 ± 0.006
Ward's triangle	0.505 ± 0.006	0.509 ± 0.005	0.507 ± 0.004	0.504 ± 0.008

Data are means ± SE
BMD values are adjusted for age and body mass index

Characteristics of female subjects are shown in Table 3. The distribution of −521C → T genotypes of *DRD4* was in Hardy-Weinberg equilibrium for women. Age was greater in women with the *TC* genotype or in the combined group of *TT* and *TC* genotypes than in women with the *CC* genotype. Height was smaller in the combined *TT-TC* group than in women with the *CC* genotype. Body weight, BMI, and the prevalence of smoking did not differ among −521C → T genotypes for women. There were no differences in BMD among −521C → T genotypes in women (Table 4). The relation of BMD to −521C → T genotype was also examined in premenopausal and postmenopausal women separately, but no association was detected (data not shown).

The relation of biochemical markers of bone turnover to −521C → T genotypes of *DRD4* was also examined (Table 5). For men, the serum concentrations of osteocalcin and bone-specific ALP did not differ among −521C → T genotypes, whereas urinary excretion of deoxypyridinoline was slightly, but significantly, greater in individuals with the *CC* genotype than in those with the *TT* genotype or in the combined group of *TT* and *TC* genotypes. For women, the serum concentrations of osteocalcin and bone-specific ALP as well as urinary

Table 5 Biochemical markers of bone turnover for men or women according to the –521C → T genotype of *DRD4*. *B-ALP* bone-specific ALP, *dPyr* deoxypyridinoline, *Cr* creatinine, *BUN* blood urea nitrogen

Marker	Men (n = 1,123)				Women (n = 1,105)			
	TT	*TC*	*TT* + *TC*	*CC*	*TT*	*TC*	*TT* + *TC*	*CC*
Osteocalcin (ng/ml)	7.69 ± 0.13	7.64 ± 0.11	7.66 ± 0.08	7.66 ± 0.19	9.32 ± 0.19	9.71 ± 0.17*	9.54 ± 0.13*	8.89 ± 0.27
B-ALP (U/l)	26.2 ± 0.4	26.0 ± 0.4	26.1 ± 0.3	25.4 ± 0.7	28.2 ± 0.6	29.5 ± 0.5*	29.0 ± 0.4*	27.0 ± 0.8
dPyr (nmol/μmol Cr)	3.99 ± 0.06*	4.01 ± 0.05	4.00 ± 0.04*	4.26 ± 0.09	6.52 ± 0.11	6.79 ± 0.10†	6.67 ± 0.07*	6.25 ± 0.16
BUN (mg/dl)	16.7 ± 0.9	16.0 ± 0.7	16.3 ± 0.5	15.3 ± 2.5	15.3 ± 0.7	14.3 ± 0.6	14.7 ± 0.5	14.5 ± 1.0
Serum Cr (mg/dl)	1.03 ± 0.02	1.02 ± 0.01	1.02 ± 0.01	0.99 ± 0.03	0.78 ± 0.01	0.77 ± 0.01	0.78 ± 0.01	0.77 ± 0.01

Data are means ± SE
*P < 0.05
†P < 0.01 versus corresponding *CC*

excretion of deoxypyridinoline were greater in those with the *TC* genotype or in the combined group of *TT* and *TC* genotypes than in those with the *CC* genotype. There were no differences in parameters for renal function, such as blood urea nitrogen or serum concentrations of creatinine, among DRD4 genotypes for men or women.

Discussion

The dopaminergic system plays an important role in the central nervous system by regulating synaptic transmission mediated by the major neurotransmitters and neuropeptides in the hypothalamus, and it performs a combined neurotransmitter-hormone function in the pituitary gland (Gilbert 1993). The effects of the dopaminergic system on the action of sex hormones, vitamin D, and parathyroid hormone may also contribute to the attainment of peak bone mass at a young age and to bone loss later in life (Gilbert 1993).

We have now shown that the –521C → T SNP of *DRD4* was significantly associated with BMD at the distal radius (D50), total body, lumbar spine, trochanter, and Ward's triangle in men, with the *CC* genotype representing a risk factor for reduced BMD. Measurement of biochemical markers of bone turnover revealed that bone resorption was slightly but significantly increased in men with the *CC* genotype. In contrast, no association between –521C → T genotype and BMD was observed for women, although women with the *TT* or *TC* genotype or with the *TC* genotype exhibited higher bone turnover. Although several gene polymorphisms have been associated with BMD in men (Gennari and Brandi 2001), the relation of genetic diversity to BMD in males has not been fully investigated. Our results suggest that *DRD4* is a candidate locus for reduced BMD in men.

The –521C → T SNP is located in one of the CpG dinucleotides in a GC-rich region of the *DRD4* promoter, but its surrounding sequence does not appear to match the recognition motif for any known transcription factor (Okuyama et al. 2000). The *C* allele of this polymorphism exhibited higher transcriptional activity than did the *T* allele in a reporter gene assay (Okuyama et al. 2000). In the present study, the *CC* genotype was associated with reduced BMD in men. Treatment with a dopamine agonist resulted in a slight increase in BMD in men with hyperprolactinemia (Di Somma et al. 1998). If the higher transcriptional activity of the *C* allele of *DRD4* results in enhanced dopaminergic activity, the association between –521C → T genotype and BMD detected in our study is thus opposite to that expected on the basis of this previous clinical study. However, differences in transcriptional activity detected in reporter gene systems do not necessarily reflect differences in the receptor characteristics in vivo. The molecular mechanism that underlies the association of the –521C → T SNP of *DRD4* with BMD in men thus remains unclear. Given that the dopamine receptor genes have not been shown to express in the bone, the dopaminergic system may not have direct actions to the bone.

The reason for the gender difference in the association of this SNP with BMD also remains to be determined. The dopaminergic system interacts with various hormones, including androgens (Gilbert 1993). Androgens influence bone development and metabolism. Decreased androgen levels have been linked to lower BMD in men, and there is a strong correlation between hypogonadism in elderly men and hip fracture or osteoporosis (Vanderschueren and Bouillon 1995). It is possible that the –521C → T polymorphism of *DRD4* affects the action of androgens in men. The gender difference might be attributable, at least in part, to the differences in the serum concentrations of androgens between men and women.

It is possible that the –521C → T SNP of *DRD4* is in linkage disequilibrium with polymorphisms of other nearby genes that are determinants of BMD. Indeed, the p57 gene (chromosome 11p15.5), parathyroid hormone gene (11p15.3–15.1), and calcitonin gene (11p15.2–15.1), all of which have been associated with BMD (Hosoi et al. 1999; Miyao et al. 2000; Urano et al. 2000), are located in close proximity to *DRD4* (11p15.5). However, our present results suggest that *DRD4* is a candidate locus for reduced bone mass in Japanese men.

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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 *ε4* allele of the apolipoprotein E gene (*APOE-ε4*) (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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Molecular Genetics and Epidemiology of Osteoporosis

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Abstract: Although various environmental factors, including diet and physical exercise, influence bone mass, a genetic contribution to this parameter has also been recognized. Genetic linkage analyses and candidate gene association studies have implicated several loci, and a variety of candidate genes in the regulation of bone mineral density and the pathogenesis of osteoporosis or osteoporotic fracture. However, the genes responsible for these latter conditions have not been identified definitively. I here summarize both the candidate loci identified by linkage analyses and the candidate genes examined by association studies. I also review in more detail studies that have examined the association with bone mass or with the susceptibility to osteoporosis or osteoporotic fracture of polymorphisms in the genes for the vitamin D receptor, collagen type Iα1, estrogen receptor α, interleukin-6, osteocalcin, osteoprotegerin, CC chemokine receptor 2, matrix metalloproteinase–1, and transforming growth factor–β1. Such studies may provide insight into the function of these genes as well as into the role of genetic factors in the development of osteoporosis.

INTRODUCTION

Bone mass is determined by various mechanisms that are affected by multiple chromosomal loci as well as by environmental factors, such as diet and physical exercise. Family and twin studies have shown that genetic factors account for most of the variance in bone mineral density (BMD) in the general population, with the inheritance of bone mass thought to be under polygenic control [Nguyen *et al.* 1994]. Osteoporosis is a common disease that is characterized by reduced bone mass and deterioration of bone architecture, both of which result in an increased risk of fracture [Kanis *et al.* 1994].

There are two basic strategies for identifying genes that influence BMD or other complex traits [Nguyen *et al.* 2000]: the candidate gene approach, in which individual genes are examined directly for a possible role in determination of the trait of interest, and the genome-wide screening approach, in which all genes are examined systematically with panels of micro-satellite DNA markers uniformly distributed throughout the genome. In each of these approaches, susceptibility genes or loci are identified by demonstration of a significant linkage or association [Nguyen *et al.* 2000]. Linkage analysis involves the proposition of a model to account for the pattern of inheritance of a phenotype observed in a pedigree. It determines whether or not the phenotypic locus is transmitted together with genetic markers of known chromosomal position. Genetic association refers to the occurrence of a certain allele or locus at a frequency higher than that expected by chance in individuals with a particular phenotype. Such an association is suggested by a statistically significant difference in the prevalence of alleles with respect to the phenotype.

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Genetic linkage analyses [Morrison *et al.* 1994; Johnson *et al.* 1997; Devoto *et al.* 1998] and candidate gene association studies [Morrison *et al.* 1994; Grant *et al.* 1996; Kobayashi *et al.* 1996; Yamada *et al.* 1998] have implicated several loci and candidate genes in the regulation of BMD and the pathogenesis of osteoporosis or osteoporotic fracture. The genes that contribute to genetic susceptibility to osteoporosis remain to be identified definitively, however. The identification of genes that confer susceptibility to osteoporotic fracture is especially important for the detection of individuals at risk for this condition and in targeting preventive treatment.

LINKAGE ANALYSES

The results of whole-genome linkage analysis [Johnson *et al.* 1997; Devoto *et al.* 1998], partial-genome linkage analysis [Devoto *et al.* 2001], and linkage analysis of candidate loci or genes [Duncan *et al.* 1999; Ota *et al.* 1999, 2000; Raymond *et al.* 1999] for BMD or osteoporosis are summarized in Table **1**. A locus linked to low BMD was mapped to chromosomal region 1p36.3-36.2 [Devoto *et al.* 2001], whereas a locus linked to high BMD was localized to 11q12-13 [Johnson *et al.* 1997]. Genes responsible for other BMD-related phenotypes, such as autosomal recessive osteoporosis-pseudoglioma syndrome [Gong *et al.* 1996] and autosomal dominant osteopetrosis [Van Hul *et al.* 2002], are also located at 11q12-13, suggesting that the genes underlying these disorders or other genes at these loci might play a role in determining BMD in the general population.

ASSOCIATION STUDIES

Polymorphisms of a variety of candidate genes have been associated with BMD or with genetic susceptibility to osteoporosis or osteoporotic fracture (Table **2**). These genes include those encoding tumor necrosis factor (TNF) receptor 2, methylenetetrahydrofolate reductase, and osteocalcin

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