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Alcohol dehydrogenase 2 variant is associated with cerebral infarction and lacunae

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Abstract—The authors examined the association of the alcohol dehydrogenase 2 (ADH2) genotype with vascular events in community-dwelling Japanese (1,102 men/1,093 women). The allele *ADH2*2* encodes an isozyme with a higher level of activity than ADH2*1. Here, the authors show that the *ADH2*1* carriage is associated with high prevalence of cerebral infarction and lacunae in men. Multiple regression analyses confirmed that the risk of lacunae and cerebral infarction was increased by the *ADH2*1* allele.

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Alcohol dehydrogenase (ADH) is one of the key enzymes in alcohol metabolism. *ADH2* and *ADH3* have alleles that encode isoenzymes with distinct enzymatic properties.[1] Among Caucasians, a variant *ADH3* allele is found. On the other hand, among Mongoloids, especially the Japanese, about 85% of individuals are carriers of the β2-subunit encoded by the *ADH2*2* allele, compared to only 5% or less of European and white American populations. The β1 (encoded by *ADH2*1*) and β2 subunits (encoded by *ADH2*2*) differ by only one amino acid residue, Arg-47 in the β1 subunit substituted with His-47 in the β2 subunit. ADH2 functions as a dimer and the β2β2 dimer exhibits about 100 times more catalytic activity than the β1β1 dimer.[1]

We previously reported on the influence of the *ADH2* and aldehyde dehydrogenase 2 genotypes on diabetic vasculopathy in type 2 diabetes.[2] Here we examined whether the *ADH2* genotype would also be associated with vascular events in community-dwelling Japanese and show the association of the *ADH2*1* allele with cerebral infarction.

Materials and methods. A population-based prospective cohort study of aging and age-related diseases was begun in Japan in 1997. All participants (1,126 men and 1,106 women) were independent residents of Aichi prefecture. Residents aged 40 to 79 years old were randomly selected from the register in cooperation with the local government. A total of over 1,000 characteristics, including medication, food and nutrition, bone mineral density, blood and urine analysis, psychological examinations, visual and auditory examinations, physical function tests and physical activities, anthropometry and body composition, and head MRI, were examined (see http://www.nils.go.jp/index-j.html).[3] The study protocol was approved by the Committee on the Ethics of Human Research of National Chubu Hospital and the National Center for Geriatrics and Gerontology. Written informed consent for the entire procedure was obtained from each participant.

Samples of DNA were isolated from peripheral blood cells. Genotypes were determined with a fluorescence-based allele-specific DNA primer-probe assay system (Toyobo Gene Analysis, Tsuruga, Japan). Brain MRI was performed using a 1.5-tesla scanner (Toshiba Visart, Tokyo). The first scanning sequence consisted of a T1-weighted sagittal series centered in the midline to define the orbitomeatal line. The second series of T1-weighted axial images and T2-weighted axial images were oriented parallel to the orbitomeatal line. Fourteen slices were taken at each examination.

A cerebral infarction was defined as a lesion more than 0.3 cm in diameter appearing as a low-signal-intensity area on T1-weighted images that was also visible as a hyperintense lesion on T2-weighted images as described.[3,4] Small lesions (<1.5 cm) were diagnosed as a lacunae. One of the authors (M.F.), a neurologist, who was blinded to the clinical status of the subjects, interpreted all MRI series.

Results. When the subjects were grouped into three according to the genotype of *ADH2*, *ADH2*2/ADH2*2 (ADH2*2/2), ADH2*2/ADH2*1 (ADH2*2/1),* and *ADH2*1/ADH2*1 (ADH2*1/1),* the distribution of the *ADH2* genotypes was in Hardy-Weinberg equilibrium. There was no significant difference in characteristics among the three genotypic groups in women (data are not shown). In contrast, in men, the level of total cholesterol (TC) and LDL-cholesterol (LDL-C) significantly differed between the *ADH2*2/2* and *ADH2*1/2* genotypic groups by multiple comparisons (table 1). Although group *ADH2*1/1* did not significantly differ in the levels of TC and LDL-C from the other groups, probably due to an insufficient number in members of group *ADH2*1/1* (5.2%), the *ADH2*1* allele tended to increase the levels of TC and LDL-C. Additionally, alcohol consumption was higher in the *ADH2*1/1* group than the other groups, whereas there was no differ-

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*Table 1 Comparison of clinical characteristics in men among ADH2*2/2, ADH2*2/1, and ADH2*1/1 genotypic groups*

	*ADH2*2/2*	*ADH2*2/1*	*ADH2*1/1*	Genotype: *p* value
No. (%)	689 (61.2)	378 (33.6)	59 (5.2)	NS
Age, y	59.4 ± 0.4	58.8 ± 0.6	58.0 ± 1.4	NS
Alcohol, g/d	28.8 ± 1.4	29.5 ± 1.9	44.5 ± 4.5	2/2 vs 1/1: p = 0.0049* 2/1 vs 1/1: p = 0.0102*
Nonsmoker & smoker, %†	21/40/39	22/40/37	24/39/37	NS
Systolic BP, mm Hg‡	120.1 ± 0.8	121.8 ± 1.0	126.1 ± 2.6	NS
Diastolic BP, mm Hg‡	74.9 ± 0.5	76.1 ± 0.6	77.3 ± 1.6	NS
Percent with hypertension§	32.6	37.0	40.7	NS
Height, cm	164.4 ± 0.2	164.7 ± 0.3	164.6 ± 0.8	NS
BMI	23.0 ± 0.1	22.8 ± 0.1	22.9 ± 0.4	NS
T-cho, mg/dL	210.1 ± 1.3	215.7 ± 1.7	217.6 ± 4.3	2/2 vs 2/1: p = 0.0231*
LDL, mg/dL	129.7 ± 1.2	135.8 ± 1.7	134.4 ± 4.2	2/2 vs 2/1: p = 0.0115*
HDL, mg/dL	57.3 ± 0.6	57.6 ± 0.8	57.4 ± 1.9	NS
TG, mg/dL	134.9 ± 3.7	130.8 ± 5.0	150.2 ± 12.4	NS
Glucose, mg/dL	105.7 ± 0.9	106.1 ± 1.2	103.9 ± 2.9	NS
HbA1c, %	5.32 ± 0.03	5.34 ± 0.04	5.33 ± 0.10	NS
Percent with diabetes	13.3	13.3	13.6	NS
Insulin, μU/mL	8.5 ± 0.2	7.8 ± 0.3	8.7 ± 0.7	NS
Estradiol, pg/mL	28.2 ± 0.4	27.1 ± 0.5	25.9 ± 1.4	NS
F-Testosterone, pg/mL	13.1 ± 0.2	13.3 ± 0.2	13.6 ± 0.5	NS
Brain examination, n (%)	n = 678	n = 367	n = 57	
Lacunal infarction	60 (8.9)	55 (15.0)	8 (14.0)	p = 0.0085¶ 2/2 vs 2/1: p = 0.0025‖
Cerebral infarction	68 (10.0)	59 (16.1)	9 (15.8)	p = 0.0129¶ 2/2 vs 2/1: p = 0.0043‖

Values are mean ± SD or n (%).

* p Value obtained by the Turkey-Kramer method for multiple comparisons.
† Nonsmoker & smoker = percentage of complete nonsmokers/percentage of past smokers who stopped smoking/percentage of current smokers.
‡ Blood pressure (BP) was analyzed only with subjects not taking oral antihypertension medications.
§ Hypertension was defined as either a systolic blood pressure of over 140 mm Hg or a diastolic blood pressure of over 90 mm Hg, or as receiving antihypertension medication.
¶ p Value obtained by the contingency table analysis.
‖ p Value by the chi-square analysis between groups *ADH2*2/2* and *ADH2*2/1*.

NS = not significant by multiple comparisons; BMI = body mass index; LDL = low-density lipoprotein; HDL = high-density lipoprotein.

ence in amounts of alcohol consumption between groups *ADH2*2/2* and *ADH2*2/1*.

A total of 1,102 male and 1,093 female subjects were examined by MRI. More striking, in men, higher frequencies of lacunae and cerebral infarction were found in the *ADH2*2/1* group than the *ADH2*2/2* group (see table 1). The frequencies of other abnormal signs on MRI did not differ among the three groups (data are not shown). In women, there was no difference in prevalence of abnormal MRI signs among the three *ADH2* genotypic groups (data not shown).

To confirm the significant difference in the frequencies of lacunae and cerebral infarction according to the *ADH2* genotype, multiple logistic analyses were performed based on 1,102 subjects with an adjustment for aging (table 2). Aging is the most significant risk for lacunae and cerebral infarction. More interestingly, OR and p values clearly indicated that the *ADH2*1* allele is a distinct risk for lacunae and cerebral infarction. Even when the effect of alcohol consumption was included, the main conclusion was not altered (see table 2).

Discussion. An influence on lacunae and cerebral infarction by the *ADH* genotype was found only in Japanese men. This discrepancy between genders may be speculated to be due to a difference in alcohol consumption. However, even when the effect of alcohol consumption was included, the main conclusion was not altered. Therefore, the effect by alcohol consumption does not seem responsible for the discrepancy between genders. Instead, ADH2 activity modulated by several hormones may be responsible for the discrepancy. In fact, experiments with ani-

Table 2 Multiple logistic analyses (number of subjects = 1,102)

	OR (95% CI)	*p* Value
Lacunar state in men		
A: Multiple logistic analyses		
ADH2 (carriage of *ADH2*1* allele)	2.16 (1.44–3.25)	0.0002
Age - 10 y	3.46 (2.69–4.45)	<0.0001
B: Multiple logistic analyses including alcohol consumption		
ADH2 (carriage of *ADH2*1* allele)	2.18 (1.49–3.38)	0.0005
Age - 10 y	3.53 (2.68–4.65)	<0.0001
Cerebral infarction in men		
A: Multiple logistic analyses		
ADH2 (carriage of *ADH2*1* allele)	2.06 (1.39–3.06)	0.0003
Age - 10 y	3.44 (2.70–4.37)	<0.0001
B: Multiple logistic analyses including alcohol consumption		
ADH2 (carriage of *ADH2*1* allele)	2.05 (1.35–3.11)	0.0008
Age - 10 y	3.49 (2.70–4.52)	<0.0001

mals indicated that testosterone reduces enzymatic activity in the liver, and that estrogen increases the activity.[5]

ADH catalyzed the first step in the metabolism of ethanol, and in addition, has a wide substrate range, using both aliphatic and aromatic alcohols, aldehydes, sterols, and ω-hydroxy fatty acids. It is worth noting that ADH catalyzes the oxidation of 3,3-dimethylallyl alcohol, the intermediary alcohol of the shunt pathway of mevalonate metabolism, and the branching between the sterol and the shunt pathway could also occur at the level of geranyl pyrophosphate and farnesyl pyrophosphate.[6] Therefore, the genetic variant of *ADH2* may change the flow of the shunt pathway of cholesterol synthesis, thereby causing LDL-C levels to vary between the *ADH2*2/2* and *ADH2*2/1* groups. As for cardiovascular diseases, it was reported that an ADH3 polymorphism is associated with HDL-C levels and myocardial infarction in Caucasians.[7] Thus, our results may provide insight into ethnic differences in the incidence of cerebral or myocardial vascular disease between Mongoloids and Caucasians.

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ORIGINAL ARTICLE

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Association of polymorphisms of the androgen receptor and klotho genes with bone mineral density in Japanese women

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Abstract Genetic variants of the androgen receptor and klotho protein may contribute to variation in bone mass as well as to predisposition to osteoporosis. The relationship of a CAG repeat polymorphism of the androgen receptor gene (*AR*) and of a −395G→A polymorphism of the klotho gene (*KL*) to bone mineral density (BMD) in Japanese women was examined in a population-based study. The subjects (1,101 and 1,110 women for *AR* and *KL* polymorphisms, respectively) 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 total body, lumbar spine, right femoral neck, right trochanter, and right Ward's triangle was measured by dual-energy X-ray absorptiometry. Genotypes for the *AR* and *KL* polymorphisms were determined by polymerase chain reaction based assays. The number of CAG repeats of *AR* was inversely correlated with BMD for the lumbar spine in premenopausal women but not in postmenopausal women. The $(CAG)_{n\leq22}$ and $(CAG)_{n\geq23}$ alleles were designated *S* and *L*, respectively. Among premenopausal women, BMD for the total body was significantly lower in subjects with the *LL* genotype than in those with the *SS* genotype or those in the combined group of *SS* and *SL* genotypes. In contrast, BMD was not associated with *AR* genotype in postmenopausal women. Among all women, BMD for the lumbar spine was significantly lower in subjects with the *GG* genotype of the −395G→A polymorphism of *KL* than in those with the *AA* genotype. BMD was not associated with −395G→A genotype among premenopausal women. In postmenopausal women, BMD for the total body or lumbar spine tended to be lower in subjects with the *GG* genotype than in those with the *AA* genotype or those in the combined group of *GA* and *AA* genotypes. These results suggest that *AR* is a susceptibility gene for reduced BMD in premenopausal Japanese women, and that *KL* is a susceptibility gene for reduced BMD in all women.

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Keywords Bone density · Androgen receptor · Klotho protein · Genetics · Osteoporosis

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Abbreviations *AR:* Androgen receptor · *BMD:* Bone mineral density · *PCR:* Polymerase chain reaction

Introduction

Osteoporosis, a major health problem of the elderly, is characterized by a reduction in bone mineral density (BMD) and a deterioration in the microarchitecture of bone, both of which result in predisposition to fractures [1]. Although reproductive, nutritional, and life-style factors influence BMD, family and twin studies have suggested that this parameter is largely heritable and under the control of multiple genes [2, 3, 4]. Genetic linkage analyses [5, 6, 7] and candidate gene association studies [8, 9, 10] have thus implicated several loci and candidate genes in the regulation of bone mass and the prevalence of osteoporosis or osteoporotic fractures. Such candidate genes include those for the androgen receptor (*AR*) and klotho [11, 12].

Androgens play important roles in the development and metabolism of bone [13]. The *AR* is expressed in human osteoblastic cells as well as in human osteoclasts, suggesting that androgens exert direct effects on bone cells [14]. The gene encoding the AR (AR), which is located on human chromosome Xq11-q12, is thus an important candidate susceptibility gene for osteoporosis. Variation in the size of the microsatellite region in the first exon of *AR* is attributable to a CAG repeat polymorphism that encodes a polyglutamine tract comprising 9–35 residues in the amino-terminal domain of the receptor protein [15, 16]. In vitro transfection assays have demonstrated that *AR* proteins with shorter polyglutamine tracts possess greater transactivation activity [17, 18, 19] whereas tract size does not affect the binding of androgens to the receptor [20]. Although the CAG repeat polymorphism of *AR* was shown to be associated with BMD in women or in men in some studies [11, 21, 22, 23, 24], other studies have failed to detect an effect of this polymorphism on BMD or fracture risk [25, 26]. Furthermore, racial differences in the number of CAG repeats have been demonstrated, with African-Americans exhibiting a higher prevalence of short CAG repeat sequences than other ethnic groups [15, 27]. Given the ethnic differences in CAG repeat length as well as in other genetic or environmental influences on BMD, it is important to examine the relationship of the CAG repeat polymorphism of *AR* to BMD in each ethnic group.

Klotho is a type I membrane protein that shares sequence similarity with members of the glycosidase family [28]. Mice deficient in this protein exhibit multiple aging phenotypes and age-related disorders, including a shortened life span, reduced spontaneous activity, arteriosclerosis, infertility, skin atrophy, premature thymic involution, pulmonary emphysema, and osteopenia, although the function of klotho remains to be determined [28]. The osteopenia observed in klotho-deficient mice is accompanied by a reduced turnover of bone: a decrease in bone formation exceeds a decrease in bone resorption, resulting in substantial bone loss that resembles that in aging humans [29]. A human homolog of the mouse klotho gene has been isolated and its structure determined [30]. The human gene (*KL*) comprises five exons and spans approx. 50 kb on chromosome 13q12. Ogata et al. [31] examined the relationship of a CA repeat polymorphism downstream of *KL* to BMD and showed that the alleles corresponding to 22 and 24 repeats are associated with low and high BMD, respectively. Kawano et al. [12] identified eight and six polymorphisms of *KL* in white and Japanese women, respectively, and showed that the −395G→A polymorphism in the promoter of *KL* is associated with BMD in postmenopausal (≥65 years) women of each ethnicity. The sizes of the populations in which this association was detected were only small (55 white, 215 Japanese), however. Large-scale population-based studies are thus required to assess the effect of this polymorphism on BMD.

We attempted to identify genes significantly associated with BMD in Japanese women in a population-based study. *AR* and *KL* are both candidates for genes that confer susceptibility to osteoporosis. We thus examined the relationship of polymorphisms of these genes to BMD in the present study, although there is no apparent biological link between the two genes. Our aim was to identify a single polymorphism significantly associated with BMD for each gene. Among several polymorphisms previously identified in *KL*, only the −395G→A polymorphism has been shown to potentially affect gene function. We therefore selected this polymorphism for our analysis. We have now examined whether the CAG repeat polymorphism of *AR* or the −395G→A polymorphism of *KL* is associated with BMD in Japanese women in a population-based study.

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 [32]. The present study represents a cross-sectional analysis within the NILS-LSA. The subjects of the NILS-LSA are stratified by both age and gender and were randomly selected from resident registrations in the city of Obu and town of Higashiura in central Japan [32, 33]. The life-style of residents of this area is typical of that of individuals in most regions of Japan. The NILS-LSA aimed to recruit equal numbers of men and women. Age at the baseline was 40–79 years, and the numbers of participants in each age decade (40s, 50s, 60s, and 70s) were similar. The planned number of participants was 2,400, that is, approx. 300 men and 300 women in each age decade. A total of 7,855 men and women was randomly selected from the community-dwelling population; of these selected individuals 16 were already deceased and 49 had moved away. The remaining 7,790 individuals were invited to attend an explanatory meeting by mail; a total of 3,434 replied, 881 of whom declined to attend the meeting, 2,553 agreed to attend, and 2,513 actually did attend. After the explanatory meeting, 2,267 individuals participated in the initial examination. Thus of the 7,790 individuals contacted by mail and the 34.34 individuals who replied, 29.1% and 66.0%, respectively, enrolled in the study. 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. Among the 2,267 participants 1,128 are women. Eighteen women who had disorders known to cause abnormalities of bone metabolism, including diabetes mellitus, renal diseases, rheumatoid arthritis, and thyroid, parathyroid, and other endocrine diseases, or who had taken drugs such as estrogen, progesterone, glucocorticoids, and bisphosphonates were excluded from the present study. Nine women whose *AR* genotype was not successfully determined were also excluded from the analysis of the relationship of the *AR* polymorphism to BMD.

We examined the relationship of BMD at various sites to the CAG repeat polymorphism of *AR* and to the −395G→A polymorphism of *KL* in 1,101 and 1,110 women, respectively. The study protocol complies with the Declaration of Helsinki and was approved by the Committee on Ethics of Human Research of National Chubu Hospital and the NILS. Written informed consent was obtained from each subject.

Measurement of BMD

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 (QDR 4500; Hologic, Bedford, Mass., USA). The coefficients of variance of the machine 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

The polymorphic region in exon 1 of *AR* was amplified by the polymerase chain reaction (PCR) with a sense primer labeled at the 5′ end with 6-carboxyfluorescein (5′-ACCTCCCGGCGCC-AGTTTG-3′) and with an antisense primer (5′-CTGCTGCTGC-CTGGGGCTAG-3′). The reaction mixture (25 µl) contained 20 ng DNA, 5 pmol of each primer, 0.2 mmol/l of each deoxynucleoside triphosphate, 2.5 mmol/l $MgSO_4$, and 0.4 U KODplus DNA polymerase (Toyobo, Osaka, Japan) in polymerase buffer. The amplification protocol comprised initial denaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 30 s and annealing-extension at 68°C for 30 s; and a final extension at 68°C for 2 min. The size of microsatellite-containing DNA fragments amplified by PCR was determined with a Prism 3100 DNA sequencer with GeneScan and Genotyper software (Applied Biosystems, Foster City, Calif., USA).

Genotypes for *KL* were determined with a fluorescence-based allele-specific DNA primer assay system [34]. The polymorphic region of *KL* was amplified by PCR with allele-specific sense primers labeled at the 5′ end with either fluorescein isothiocyanate (5′-GGCGCCCGACCAACTTXCC-3′) or Texas red (5′-GGCGCC-GACCAACTTXTC-3′) and with an antisense primer labeled at the 5′ end with biotin (5′-CTAGGGCCCGGCAGGATC-3′). The reaction mixture (25 µl) contained 20 ng DNA, 5 pmol of each primer, 0.2 mmol/l of each deoxynucleoside triphosphate, 2.5 mmol/l $MgCl_2$, and 1 U of rTaq DNA polymerase (Toyobo) in polymerase buffer. The amplification protocol comprised initial denaturation at 95°C for 5 min; 40 cycles of denaturation at 95°C for 30 s, annealing at 65°C 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

Since quantitative data were not necessarily all distributed normally, they were compared by both parametric and nonparametric tests. Comparisons between two groups were performed with the unpaired Student's *t* test or the Mann-Whitney *U* test, and those among three or more groups were compared by one-way analysis of variance and the Tukey-Kramer post hoc test or by the Kruskal-Wallis test (SAS, SAS Institute, Cary, N.C., USA). Since the results obtained with parametric and nonparametric tests were similar, statistical analyses with the former are shown in Tables 1, 2, 3, and 4. BMD values were analyzed with adjustment for age, height, and body weight by the least squares method in a general linear model. Allele frequencies were estimated by the gene-counting method, and the χ^2 test was used to identify significant departure from Hardy-Weinberg equilibrium. The effects of the CAG repeat genotype of *AR*, the −395 G→A genotype of *KL*, or both genotypes on BMD at various sites for all women were evaluated by regression analysis; R^2 and *P* values were calculated from analysis of *AR* genotype and/or *KL* genotype. We considered a *P* value of 0.005 or less to be statistically significant for the multiple comparisons of genotypes with BMD. For other background data, a *P* value of 0.05 or less was considered statistically significant. We also calculated the statistical power to detect differences in BMD among women with different genotypes, where α=0.0167 among three groups, α=0.0083 among four groups, and β=0.1.

Results

The distribution of the number of CAG repeats in *AR* for all women ranged from 12 to 37 (22.8±2.9; Fig. 1). The number of CAG repeats was significantly related to L2–L4 BMD for premenopausal women, but not for postmenopausal or total women (Fig. 2). Among premenopausal women BMD for the lumbar spine decreased as the number of CAG repeats increased. Since the mean number of CAG repeats was 22.8, we designated $(CAG)_{n\leq22}$ and $(CAG)_{n\geq23}$ alleles as short (*S*) and long (*L*) alleles, respectively.

The distributions of *SS*, *SL*, and *LL* genotypes of *AR* were in Hardy-Weinberg equilibrium, and age, height,

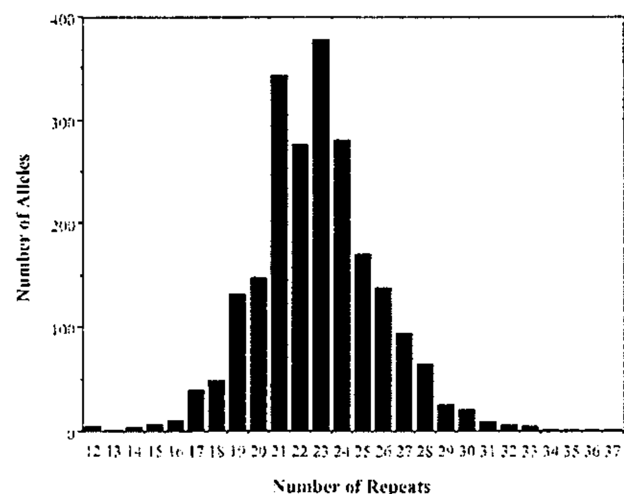

Fig. 1 Distribution of the number of CAG repeats in *AR* in 1,101 women (2,202 alleles)

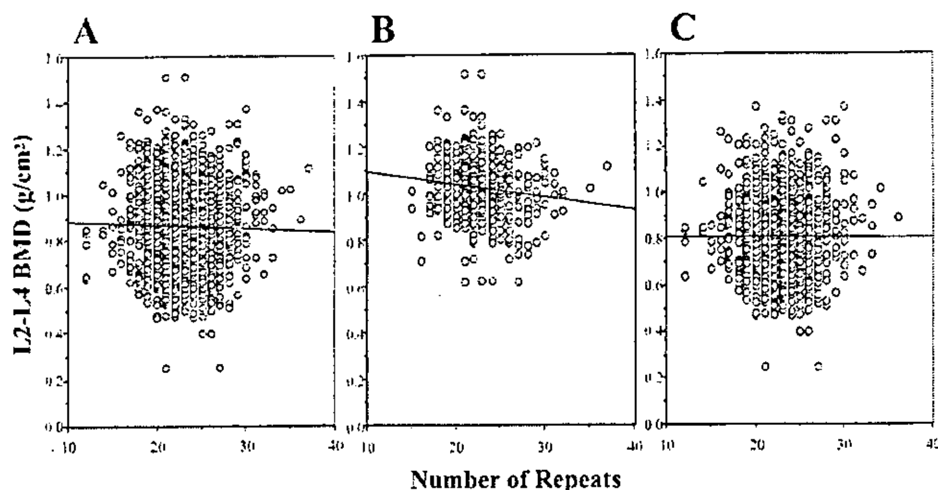

Fig. 2 Relationship between the number of CAG repeats in *AR* and L2-L4 BMD. **A** All women (n=1,101, 2,202 alleles); r=−0.01967, P=0.3584. **B** Premenopausal women (n=275, 550 alleles); r=−0.14455, P=0.0007. **C** Postmenopausal women (n=809, 1,618 alleles); r=0.00751, P=0.7644

Table 1 BMD and other characteristics of all women (n=1,101) according to the CAG repeat genotype of *AR*. BMD values are adjusted for age, height, and body weight

	SS (n=238, 21.6%)	*SL* (n=535, 48.6%)	*LL* (n=328, 29.8%)	*SS* + *SL* (n=773, 70.2%)	*SL* + *LL* (n=863, 78.4%)
Age (years)	58.9±0.7	59.1±0.5	59.9±0.6	59.1±0.4	59.4±0.4
Height (cm)	151.8±0.4	151.2±0.3	151.0±0.3	151.4±0.2	151.1±0.2
Body weight (kg)	52.3±0.5	52.4±0.4	52.6±0.5	52.4±0.3	52.5±0.3
BMD (g/cm^2)					
Total body	0.972±0.006	0.965±0.004	0.961±0.005	0.967±0.003	0.963±0.003
L2–L4	0.884±0.008	0.861±0.005*	0.860±0.007	0.868±0.005	0.860±0.004**
Femoral neck	0.686±0.006	0.677±0.004	0.675±0.005	0.680±0.003	0.676±0.003
Trochanter	0.576±0.005	0.570±0.004	0.568±0.005	0.572±0.003	0.569±0.003
Ward's triangle	0.514±0.008	0.506±0.005	0.505±0.006	0.508±0.004	0.506±0.004

* P≤0.05, ** P≤0.01 vs. *SS* (statistical power to detect differences in BMD among women with *SS*, *SL*, or *LL* genotypes is 0.1% of the largest value)

and body weight did not differ among genotypes, for all women (Table 1). BMD for the lumbar spine with adjustment for age, height, and body weight tended to be lower in the combined group of women with the *SL* or *LL* genotypes or in women with the *SL* genotype than in those with the *SS* genotype; the P values for these differences, however, did not achieve statistical significance.

To examine the possible influence of menopause on the relationship between genotype and BMD, we analyzed BMD and other characteristics for premenopausal and postmenopausal women independently. Because of their small number (n=17) perimenopausal women were excluded from the analysis. The distributions of *SS*, *SL*, and *LL* genotypes of *AR* were in Hardy-Weinberg equilibrium, and age, height, and body weight did not differ among genotypes, for premenopausal or postmenopausal women (Table 2). For premenopausal women, BMD for the total body was significantly (P≤0.005) lower in those with the *LL* genotype than in those with the *SS* genotype or those in the combined group of *SS* and *SL* genotypes. The difference in BMD for the total body between the *SS* genotype and the *LL* genotype was 3.9% (expressed as a proportion of the larger value). In contrast, BMD was not associated with *AR* genotype in postmenopausal women.

The distribution of −395G→A genotypes of *KL* was in Hardy-Weinberg equilibrium, and age, height, and body weight did not differ among genotypes for all women (Table 3). BMD for the lumbar spine was significantly (P≤0.005) lower in women with the *GG* genotype than in those with the *AA* genotype; the difference in L2–L4 BMD between these two groups (expressed as a percentage of the larger value) was 7.9%.

We also analyzed the relationship of BMD and other characteristics to *KL* genotype for premenopausal and postmenopausal women independently (Table 4). The distributions of −395G→A genotypes of *KL* were in Hardy-Weinberg equilibrium, and age and body weight did not differ among genotypes in premenopausal or postmenopausal women. Height did not differ among *KL* genotypes in premenopausal women, but postmenopausal women with the *GG* genotype were taller than were those with the *GA* genotype or those in the combined group of *GA* and *AA* genotypes. In premenopausal women, BMD was not associated with −395G→A genotype. In postmenopausal women, although there was a trend (P≤0.05) for BMD for the total body or lumbar spine to be lower in subjects with the *GG* genotype than in those with the *AA* genotype or those in the combined group of *GA* and *AA*

Table 2 BMD and other characteristics of women (n=1,084) according to menopausal status and the CAG repeat genotype of *AR*. BMD values are adjusted for age, height, and body weight

	Premenopausal women (n=275)				Postmenopausal women (n=809)			
	SS (n=62, 22.6%)	*SL* (n=134, 48.7%)	*LL* (n=79, 28.7%)	*SS* + *SL* (n=196, 71.3%)	*SS* (n=173, 21.4%)	*SL* (n=393, 48.6%)	*LL* (n=243, 30.0%)	*SS* + *SL* (n=566, 70.0%)
Age (years)	46.2±0.6	46.0±0.4	46.6±0.5	46.0±0.3	63.6±0.7	63.8±0.4	64.4±0.6	63.7±0.4
Height (cm)	154.4±0.6	154.4±0.4	154.5±0.5	154.4±0.3	150.8±0.5	150.0±0.3	149.8±0.4	150.3±0.3
Body weight (kg)	53.9±1.0	54.4±0.7	54.6±0.9	54.2±0.6	51.7±0.6	51.7±0.4	51.8±0.5	51.7±0.3
BMD (g/cm^2)								
Total body	1.111±0.010	1.102±0.007*	1.068±0.009**, *****	1.105±0.006	0.922±0.007	0.916±0.004	0.921±0.006	0.918±0.004
L2–L4	1.050±0.014	1.031±0.010	0.997±0.013****	1.037±0.008	0.826±0.010	0.801±0.006	0.809±0.008	0.809±0.005
Femoral neck	0.780±0.011	0.777±0.008	0.762±0.010	0.778±0.006	0.654±0.006	0.640±0.004	0.643±0.005	0.645±0.004
Trochanter	0.668±0.010	0.664±0.007	0.642±0.009***	0.665±0.006	0.544±0.006	0.537±0.004	0.541±0.005	0.539±0.003
Ward's triangle	0.674±0.015	0.666±0.010	0.641±0.013	0.668±0.008	0.457±0.009	0.449±0.006	0.456±0.007	0.452±0.005

*P≤0.01, **P≤0.005 vs. *SS*, ***P≤0.05, [4]*§P≤0.01, [5]*P≤0.001 vs. *SS* + *SL* (statistical power to detect differences in BMD among premenopausal or postmenopausal women with *SS*, *SL*, or *LL* genotypes is 0.2% or 0.1% of the largest value, respectively)

Table 3 BMD and other characteristics in all women (n=1,110) according to the −395G→A genotype of *KL*. BMD values are adjusted for age, height, and body weight

	GG (n=812, 73.2%)	*GA* (n=268, 24.1%)	*AA* (n=30, 2.7%)	*GA* + *AA* (n=298, 26.8%)
Age (years)	59.4±0.4	58.9±0.7	58.8±2.0	58.9±0.6
Height (cm)	151.5±0.2	150.7±0.4	151.0±1.1	150.7±0.4
Body weight (kg)	52.5±0.3	52.1±0.5	53.2±1.5	52.2±0.5
BMD (g/cm^2)				
Total body	0.962±0.003	0.970±0.005	0.994±0.016	0.973±0.005
L2–L4	0.860±0.004	0.872±0.008	0.934±0.023**,***	0.878±0.007*
Femoral neck	0.678±0.003	0.675±0.005	0.692±0.016	0.677±0.005
Trochanter	0.569±0.003	0.572±0.005	0.601±0.015	0.575±0.005
Ward's triangle	0.504±0.004	0.511±0.007	0.537±0.021	0.513±0.007

* P≤0.05, **P≤0.005 vs. *GG*, ***P≤0.05 vs. *GA* (statistical power to detect differences in BMD among women with *GG*, *GA*, or *AA* genotypes is 0.1% of the largest value)

Table 4 BMD and other characteristics in women (n=1093) according to menopausal status and the −395G→A genotype of *KL*. BMD values are adjusted for age, height, and body weight.

	Premenopausal women (n=278)				Postmenopausal women (n=815)			
	GG (n=199, 71.6%)	*GA* (n=71, 25.5%)	*AA* (n=8, 2.9%)	*GA* + *AA* (n=79, 28.4%)	*GG* (n=602, 73.9%)	*GA* (n=191, 23.4%)	*AA* (n=22, 2.7%)	*GA* + *AA* (n=213, 26.1%)
Age (years)	46.3±0.3	46.0±0.5	45.5±1.6	45.9±0.5	63.9±0.3	64.0±0.6	63.6±1.8	63.9±0.6
Height (cm)	154.4±0.3	154.7±0.6	152.9±1.7	154.5±0.5	150.5±0.2	149.1±0.4*	150.4±1.3	149.2±0.4**
Body weight (kg)	54.4±0.6	53.8±1.0	55.0±2.9	53.9±0.9	51.9±0.3	51.4±0.6	52.5±1.7	51.5±0.6
BMD (g/cm^2)								
Total body	1.094±0.006	1.087±0.010	1.133±0.029	1.092±0.009	0.914±0.004	0.928±0.006	0.946±0.018	0.930±0.006*
L2–L4	1.023±0.008	1.023±0.013	1.110±0.040	1.032±0.013	0.803±0.005	0.818±0.009	0.874±0.027*	0.824±0.009*
Femoral neck	0.774±0.006	0.765±0.011	0.781±0.032	0.767±0.010	0.643±0.003	0.643±0.006	0.662±0.018	0.645±0.006
Trochanter	0.661±0.006	0.646±0.010	0.684±0.029	0.650±0.009	0.536±0.003	0.547±0.006	0.572±0.017	0.549±0.006
Ward's triangle	0.656±0.008	0.658±0.014	0.714±0.042	0.664±0.013	0.450±0.005	0.458±0.008	0.475±0.025	0.459±0.008

* P≤0.05, **P≤0.01 vs. *GG* (statistical power to detect differences in BMD among premenopausal or postmenopausal women with *GG*, *GA*, or *AA* genotypes is 0.2% or 0.1% of the largest value, respectively)

Table 5 Effects of the CAG repeat genotype of *AR*, the −395G→A genotype of *KL*, or both genotypes on BMD in all women (n=1,110). The R^2 and P values were derived from regression analysis of *AR* genotype (0=*SS*, 1=*SL*=*LL*) and/or KL genotype (0=*GG*=*GA*, 1=*AA*)

	AR genotype		*KL* genotype		*AR* and *KL* genotypes	
	R^2	P	R^2	P	R^2	P
Total body						
AR	0.0023	0.1255	0.0015	0.2151	0.0026	0.1016
KL					0.0015	0.2157
L2–L4						
AR	0.0045	0.0307	0.0045	0.0287	0.0048	0.0256
KL					0.0046	0.0281
Femoral neck						
AR	0.0031	0.0735	0.0008	0.3457	0.0034	0.0621
KL					0.0008	0.3464
Trochanter						
AR	0.0013	0.2399	0.0027	0.0921	0.0016	0.1991
KL					0.0027	0.0958
Ward's triangle						
AR	0.0015	0.2124	0.0013	0.2382	0.0017	0.1856
KL					0.0013	0.2432

genotypes, the P values for these relationships did not achieve statistical significance.

Finally, the effects of the CAG repeat genotype of *AR*, the −395G—A genotype of *KL*, or both genotypes on BMD at various sites in all women were evaluated by regression analysis (Table 5). Although there was a trend ($P \leq 0.05$) that *AR* genotype and *KL* genotype affected BMD for the lumbar spine, this difference was not statistically significant. The effects of the two polymorphisms on BMD were statistically independent.

Discussion

The CAG repeat polymorphism of *AR* has previously been shown to be associated with osteoporosis in men. In a study of white men, repeat length was inversely correlated with BMD, with long repeats [$(CAG)_{n>21}$] being associated with lower phalangeal BMD, higher bone turnover, and increased bone loss [21]. A study of Finnish men, however, did not detect an association between this polymorphism of *AR* and BMD [26]. In women overrepresentation of certain *AR* genotypes (combinations of alleles with 22, 23, 24, or 25 repeats) was found among pre- or perimenopausal individuals with low BMD [11]. A Danish study demonstrated a higher frequency of long alleles in women with osteoporotic fractures and a negative correlation between allele size and BMD [22]. In contrast, no association was observed between the *AR* polymorphism and BMD in a study of Finnish women [25]. The effects of the CAG repeat polymorphism of *AR* on BMD have not previously been determined for premenopausal and postmenopausal women independently in the same ethnic group.

We have now shown that the number of CAG repeats in *AR* is inversely correlated with BMD for the lumbar spine in premenopausal Japanese women, and that BMD for the total body is significantly lower in premenopausal women with two $(CAG)_{n\geq23}$ alleles than in those with one or two $(CAG)_{n\leq22}$ alleles. Our observation that long repeat alleles are associated with reduced BMD is consistent with the similar previous observation in Danish women [22].

This association between BMD and the CAG repeat polymorphism is possibly attributable to the fact that the transactivation activity of the *AR* is inversely correlated with the number of CAG repeats [17, 18, 19]. In vitro observations thus suggested that a decrease of six CAG repeats results in a 12% increase in ligand-dependent transactivation activity of the *AR* [18]. This relationship between repeat length and transactivation activity is due in part to variation in the basal activity of the *AR* and to functional interaction of the polyglutamine tract with coactivators [35, 36]. In addition, the serum concentration of androgens is related to the CAG repeat polymorphism of *AR*, with short alleles being associated with higher levels of androgens in premenopausal women [37]. This finding supports our observation that the *AR* polymorphism is associated with BMD in premenopausal, but not postmenopausal, women, although the definition of short alleles differed between this previous study [$(CAG)_{n\leq19}$] [37] and our study [$(CAG)_{n\leq22}$] and postmenopausal women were not examined in the previous study [37].

The mean number of CAG repeats for the *AR* in our population (22.8) was greater than that previously reported in Danish women (21.9) [24] or in Danish normal (20.5) or osteoporotic (21.0) women [22]. Furthermore, the mean number of CAG repeats in African-American men (20.1) was smaller than that in white men (22.1) or Asian men (22.1) [15]. These differences in repeat number may account at least in part for the differences in BMD or in the prevalence of osteoporosis among ethnic groups. Since the mean number of CAG repeats was 22.8 in our study population, we designated $(CAG)_{n\leq22}$ and $(CAG)_{n\geq23}$ alleles as short (S) and long (L) alleles, respectively. The cutoff value for the CAG repeat number in our study was thus greater than that in previous studies: $(CAG)_{n\leq21}$ [24], $(CAG)_{n\leq20}$ [22], $(CAG)_{n\leq19}$ [37], and $(CAG)_{n\leq18}$ [25] for the S allele.

The somatic cells of most females contain two X chromosomes, only one of which is active. The process of X chromosome inactivation, which occurs early in de-

velopment, is usually random, resulting in the generation of tissues with approximately equal numbers of cells in which the active X chromosome is of maternal or paternal origin [38]. Deviation from such an equal distribution of the two cell types can occur, however. A skewed pattern of X chromosome inactivation affecting the CAG repeat polymorphism of *AR* has been associated with other hormone-related diseases in women [38, 39, 40]. Given that no information is available on the relative extents of inactivation of the *S* and *L* alleles of *AR* in the present study, the evaluation of BMD in individuals with the *SL* genotype requires caution.

The −395G→A and 1818C→T polymorphisms of *KL* have previously been associated with BMD for the total body in white women aged 65 years or older and with that for the distal radius in Japanese women of the same age group, with BMD decreasing according to the rank orders of genotypes *GG* >*GA* >*AA* for the −395G→A polymorphism and *CC* >*CT* >*TT* for the 1818C→T polymorphism [12]. In the present study we examined the relationship of BMD at various sites to the −395G→A polymorphism but not to the 1818C→T polymorphism, since the latter is a synonymous polymorphism (His→His) and appears not to have a functional effect. We found that the −395G→A polymorphism of *KL* is significantly associated with BMD for the lumbar spine in all women, with the *GG* genotype representing a risk factor for reduced BMD. However, when premenopausal and postmenopausal women were analyzed separately, this polymorphism was not significantly related to BMD in either group, although there was a trend for the *GG* genotype to be associated with low BMD in postmenopausal women. The alleles of the −395G→A polymorphism associated with reduced BMD thus differ between the present study (*G* allele) and the previous study (*A* allele) [12]. Although the reason for this discrepancy is unclear, there are two major differences between the two studies: (a) The number of subjects in which the association was detected was greater in our study (n=1,110) than in the previous study (n=55 for white women, n=215 for Japanese women). (b) BMD was compared among *KL* genotypes with adjustment for age, height, and body weight in our study, but BMD was not adjusted in the previous study. However, it is possible that the −395G→A polymorphism of *KL* is in linkage disequilibrium with other polymorphisms of *KL* or of nearby genes that are actually the determinants of BMD. Although we adopted a strict criterion of statistical significance ($P \leq 0.005$) for the association of genotypes with BMD, we cannot completely exclude the possibility of statistical errors such as false positives.

Evidence suggests that the −395G→A polymorphism of *KL* affects promoter function [12]. Electrophoretic mobility-shift analysis revealed that the amount of DNA-protein complex formed by the *G* allele of the promoter was greater than that formed by the *A* allele, suggesting that the binding of one or more proteins to the promoter is impaired by the G→A substitution, which may affect the expression of *KL*. The effect of this polymorphism on the transcriptional activity of *KL*, however, remains to be determined.

There were no subjects with clinical vitamin D deficiency such as osteomalacia in the present population. However, National Nutrition Survey in 2001 suggested that in approximately 25% of Japanese individuals, the amount of vitamin D taken was smaller than that of daily requirement (100 IU). Serum concentrations of free thyroxine in three subjects (0.3%) slightly exceeded the normal range (0.77–1.93 ng/dl). It is thus possible that subclinical vitamin D deficiency or thyrotoxicosis affected the results obtained in the present study.

In conclusion, our present results suggest that *AR* is a determinant of BMD in premenopausal Japanese women, with the $(CAG)_{n \geq 23}$ allele representing a risk factor for reduced BMD. *KL* is also a determinant for bone mass in Japanese women, with the *G* allele being a risk factor for reduced BMD. The effects of both polymorphisms on BMD were statistically independent.

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ASSOCIATION OF CHOLECYSTOKININ-A RECEPTOR GENE POLYMORPHISM WITH ALCOHOL DEPENDENCE IN A JAPANESE POPULATION

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Abstract — Aims: Cholecystokinin (CCK), one of the most abundant neurotransmitter peptides, interacts with dopamine. Dopaminergic neurotransmission between the ventral tegmental area and the limbic forebrain is a critical neurobiological component of alcohol and drug self-administration. CCK modulates dopamine release in the nucleus accumbens via the CCK-A receptor (R). We recently determined the transcriptional start site of the human CCK-AR gene, and detected two sequence changes (–81A/G and –128G/T) in the promoter region. The aims of the present study were to determine the prevalence of the –81A/G and –128G/T polymorphism of the CCK-AR gene between alcoholics and normal control subjects and the occurrences of the polymorphisms in subtypes of alcoholics. **Methods:** The above polymorphisms were examined in 435 alcoholics and 1490 control subjects. We excluded subjects with inactive ALDH2 and employed the subjects with ALDH2*1/2*1 (384 alcoholics and 792 controls). **Results:** The allelic frequency of –81G in the CCK-AR gene polymorphism (–81A/G) was significantly higher in alcoholics than in control subjects. However, there were no differences between the two groups with respect to the frequency of –128G/T. Alcoholic patients with antisocial personality disorder and with first-degree alcoholic relatives were significantly associated with a higher frequency of the –81G allele. In addition, the age of onset of alcohol dependence was significantly earlier in patients with this allele. **Conclusions:** The CCK-AR gene –81A/G polymorphism, especially in the –81G allele, may be associated with intractable alcoholism.

INTRODUCTION

Alcohol-related behaviours and/or alcohol sensitivities are associated with the actions of various neurotransmitters and neuropeptides such as dopamine (Kalivas, 1993; Self and Nestler, 1995). Cholecystokinin (CCK), one of the most abundant neurotransmitter peptides in the brain, is known to interact with dopamine (Crawley, 1991; Marshall *et al.*, 1991; Woodruff *et al.*, 1991; Ladurelle *et al.*, 1994; Hamilton and Freeman, 1995). Thus far, two types of CCK receptors (R) (types A and B) have been cloned (Wank, 1995). Although CCK-BR is widely distributed throughout the central nervous system, CCK-AR is found in specific regions, such as the amygdala, nucleus tractus solitarius, posterior nucleus accumbens, ventral tegmental area, substantia nigra, and raphe nucleus. CCK coexists in the mesolimbic dopamine neurons, and CCK-AR mediates the release of dopamine in the nucleus accumbens (Crawley, 1991; Marshall *et al.*, 1991; Woodruff *et al.*, 1991; Ladurelle *et al.*, 1994; Hamilton and Freeman, 1995; Wank, 1995). The dopaminergic neurotransmission between the ventral tegmental area and the limbic forebrain is a critical neurobiological component of self-administration of alcohol and drugs (Kalivas, 1993; Self and Nestler, 1995).

Recent reports (Blum *et al.*, 1990; Muramatsu *et al.*, 1996) in human subjects showed an association of polymorphisms of the dopamine D2 and/or D4 receptor gene with alcohol dependence, although results have been equivocal. In contrast, Okubo *et al.* (2000) reported that the CCK gene polymorphism does not play a major role in alcohol withdrawal symptoms. Based on our recent finding of two sequence changes in the promoter region (a G to T change in nucleotide –128 and an A to G change in nucleotide –81; GenBank database accession number D85606; Funakoshi *et al.*, 2000), in the present study, we examined the association between CCK-AR gene polymorphisms and alcohol dependence.

Liver mitochondrial aldehyde dehydrogenase-2 (ALDH2) is responsible for metabolizing the acetaldehyde produced from ethanol into acetate. More than 40% of Asians have the inactive form of ALDH2, encoded either as heterozygous ALDH2*1/2*2 or homozygous ALDH2*2 (Higuchi *et al.*, 1995), while the majority of Caucasians possess the active form of ALDH2 (2*1/2*1). A previous report (Murayama *et al.*, 1998) showed that the clinical characteristics of alcoholic patients having inactive ALDH2 differed from those of alcoholic patients with active ALDH2. In this study, we excluded subjects with inactive ALDH2 to avoid the influence of its overwhelming effect as a negative risk factor for alcoholism.

SUBJECTS AND METHODS

Subjects

This study was approved by the ethics committees of the National Alcoholism Center, Kurihama Hospital, of the National Institute of Longevity Sciences (NILS) and of the Tokyo Metropolitan Institute of Gerontology. Written informed consent was obtained from each subject.

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The subjects consisted of 435 (aged 32–74 years) Japanese male alcoholics who had been consecutively hospitalized at Kurihama Hospital. They were diagnosed as having DSM-III-R (American Psychiatric Association, 1987) alcohol dependence, based on the Structured Clinical Interview for DSM-III-R (SCID) assessment (Spitzer *et al.*, 1990).

The age-matched control subjects consisted of 1134 male participants in the NILS Longitudinal Study of Aging (LSA) (Shimokata *et al.*, 2000) and 356 males who were Institute employees. They were free of alcohol dependence, based on the results of the Kurihama Alcoholism Screening Test, the most widely used alcoholism screening test in Japan, which was administered to potential controls before entering into this study.

First, the genotype of the ALDH2 gene was determined by mismatched polymerase chain reaction restriction fragment length polymorphism (PCR–RFLP) method reported previously (Kamino *et al.*, 2000). Then the CCK-AR gene polymorphism was determined in the subjects with ALDH2*1/2*1 (384 alcoholics and 792 controls).

Genotyping procedures

The polymorphism in the promoter region of CCK-AR gene was examined using a mismatched PCR–RFLP method (Funakoshi *et al.*, 2000). In brief, a pair of primers (sense primer = 5′-CATATGTACACATGTGTGTAAAAAGCAGCC-AGAC-3′ and anti-sense primer = 5′-GCCCTTTCCTGGG-CCAGACT-3′), were designed to amplify the 103-bp product, which was subsequently digested with restriction enzyme *Hin*fI, and analysed by 12% polyacrylamide gel electrophoresis. Six genotypes were identified: a wild type (–81A/A, –128G/G); heterozygous mutant types (–81A/G, –128G/G), (–81A/G, –128G/T), (–81G/G, –128G/G), (–81G/G, –128G/T); and a homozygous mutant type (–81G/G, –128T/T).

Clinical data

We used a structured clinical interview for DSM-III-R to diagnose alcohol dependence and antisocial personality disorder (Spitzer *et al.*, 1990). We also used a structured interview to obtain responses to questions on social background as well as history of drinking and alcohol withdrawal. Family histories of alcohol dependence among all biological first-degree relatives were evaluated by using the Family History Research Diagnostic Criteria (Andreasen *et al.*, 1977). Age at onset of alcoholism was defined as the age at which the individual first met the DSM-III-R diagnostic criteria for alcohol dependence.

Statistical analyses

Statistical differences between alcohol-dependent and control subjects were assessed using the chi-squared test. A continuity correction was performed when the frequency of at least one cell was less than 5. An odds ratio (OR) with a 95% confidence interval (CI) was calculated to evaluate the genotype frequencies between groups. Probability differences of $P < 0.05$ were considered statistically significant. To assess the linkage disequilibrium between the two polymorphisms of the CCK-AR gene, we calculated the D value and its significance, using the ASSOCIAT program (downloaded from the website of J. Ott: ftp://linkage.rockefeller.edu/ software/utilities/). All statistical computations were carried out using the Statistical Analysis System package, version 6.12 (SAS Institute, 1998).

RESULTS

The frequency of a wild-type (–81A/A, –128G/G) genotype was lower in alcoholics than in controls, though the difference was not significant ($P = 0.053$) (Table 1). These polymorphisms were in linkage disequilibrium and in Hardy–Weinberg equilibrium. The genotypes of (–81A/A, –128G/T), (–81A/A, –128T/T), and (–81A/G, –128T/T) were not detected. These were not detected in our previous reports, either (Funakoshi *et al.*, 2000; Shimokata *et al.*, 2000).

When the allelic frequencies were estimated, significant differences in that of the –81A/G were detected between alcoholics and controls, as shown in Table 2 ($P = 0.023$). However, the frequencies of the –128 G to T change were not significantly different between the two groups.

Based on the finding that the allelic frequency of –81G was significantly higher in alcoholics than in controls (Table 2), the association between CCK-AR gene –81A/G polymorphism and the clinical features of alcoholics was assessed (Table 3). Comparison of the genotype distributions of the CCK-AR gene –81A/G polymorphism in alcoholics and control subjects revealed that the frequencies of –81A/A were quite similar among the subgroups of alcoholic patients with

Table 1. Distribution of CCK-AR gene –81A/G, –128G/T polymorphisms in alcoholics and control subjects (participants had ALDH2*1/2*1 genotype)

Genotype	Alcoholics ($n = 384$) n (%)	Control subjects ($n = 792$) n (%)
–81A/A, –128G/G	205 (53.3)	470 (59.3)
–81A/G, –128G/G	75 (18.8)	111 (14.0)
–81A/G, –128G/T	76 (19.8)	168 (21.2)
–81G/G, –128G/G	6 (1.6)	9 (1.1)
–81G/G, –128G/T	16 (4.2)	19 (2.4)
–81G/G, –128T/T	9 (2.3)	15 (1.9)

Percentages may not total 100 due to rounding up. The difference between the wild-type genotype and the mutations (the sum of the five different types) was tested by 2 × 2 chi-squared test. $\chi^2 = 3.75$, d.f. = 1, $P = 0.053$.

Table 2. Allele frequencies of CCK-AR gene –81A/G, –128G/T polymorphisms in alcoholics and control subjects (participants had ALDH2*1/2*1 genotype)

Allele	Alcoholics ($n = 768$) n (%)	Control subjects ($n = 1584$) n (%)
–81A	*558 (72.7)	1219 (77.0)
G	210 (27.3)	365 (23.0)
–128G	658 (85.7)	1367 (86.3)
T	110 (14.3)	213 (13.7)

Percentages may not total 100 due to rounding up. $\chi^2 = 5.12$, d.f. = 1, *$P < 0.023$ for the –81A/G polymorphism. Odds ratio = 1.26. There were no differences with respect to –128G/T.

Table 3. Clinical characteristics of alcoholics with CCK-AR gene −81A/G polymorphism (participants had ALDH2*1/2*1 genotype)

Parameter	Genotype of the CCK-AR Gene −81A/G Polymorphism		
	A/A n (%)	A/G + G/G n (%)	2 × 2 table χ^2 test
Antisocial personality disorder (ASP)			
Negative	204 (54.4)	171 (45.6)	$\chi^2 = 4.99$, d.f. = 1, $P = 0.025$
Positive	1 (11.1)	8 (88.9)	(continuity adjusted)
Delirium tremens			
Negative	142 (56.8)	108 (43.2)	$\chi^2 = 3.36$, d.f. = 1,
Positive	63 (47.0)	71 (53)	$P = 0.067$
First-degree relatives			
Negative	191 (55.7)	152 (44.3)	$\chi^2 = 6.83$, d.f. = 1,
Positive	14 (34.2)	27 (65.9)	$P = 0.009$
Age of onset of alcohol dependence	41.8 ± 10.7	38.9 ± 10.7	$t = 2.54$, d.f. = 361, $P = 0.012$

Percentages may not total 100 due to rounding up.

negative ASP, with negative delirium tremens, and with negative first-degree relatives, and the control group (55.7, 57.5, and 56.9% for respective subgroups of alcoholics versus 59.3% for control subjects, as shown in Table 1). A comparison among alcoholic subgroups revealed that the frequency of genotype −81A/A was significantly lower in alcoholics with ASP and with first-degree relatives than in those without ASP and without family history (Table 3). The frequency of −81A/A tended to be lower in alcoholics with delirium tremens than in those without delirium tremens, though the difference was not significant ($P = 0.067$). The age at onset of alcohol dependence was significantly earlier in alcoholics with genotypes −81A/G and G/G than in those with wild-type (−81A/A).

DISCUSSION

Our results showed a higher frequency of the G allele of the CCK-AR gene −81A/G polymorphism in alcoholics than in control subjects. Moreover, the allelic frequency of −81G was significantly higher in alcoholic patients with ASP and with family history than in those without ASP and family history. Patients with delirium tremens tended to possess the −81G allele more frequently than did patients without delirium tremens, although the difference was not statistically significant ($P = 0.067$). Furthermore, the age at onset of alcohol dependence was earlier in patients with the −81G allele than in those without it. These findings suggest that the −81G allele of the CCK-AR gene may be associated with intractable alcohol dependence.

The comorbidity rate of antisocial personality disorder was only 2.3% and an average age at onset of alcohol dependence was around 40 years in our samples. These figures are substantially different from those of US alcoholic samples recruited from inpatient treatment settings (Hesselbrock *et al.*, 1986; Raimo *et al.*, 1999). Although reasons are not clear, we have observed a relatively low comorbidity rate of antisocial personality disorder in Japanese alcoholic samples (Yoshino and Kato, 1996; Murayama *et al.*, 1998). In addition, age of onset of our alcoholic samples is comparable to that of other Japanese alcoholic inpatients. (Murayama *et al.*, 1998). These comparisons suggest that our samples did not deviate from general Japanese alcoholic samples.

There have been several previous reports of CCK-AR gene polymorphisms (Inoue *et al.*, 1997; Tachikawa *et al.*, 2000; Okubo *et al.*, 2002). Okubo *et al.* (2002) determined five mutations, −388 $(GT)_8/(GT)_9$, −333G/T, −286A/G, −241G/A, and −85C/G in the promoter region of the CCK-AR gene, and reported a significant association between −85C to G change and alcoholic patients with hallucinations. However, once we had determined the transcriptional start site of the CCK-AR gene (Funakoshi *et al.*, 2000), we discovered that the −85 is not in the promoter region, but is in the 5′ untranslated region. Okubo *et al.* (2002) numbered not from the transcriptional start site but from the initial site of the coding region of exon 1. We examined CCK-AR gene polymorphisms in 50 patients with gallstone and 300 patients with diabetes mellitus before the establishment of the RFLP method (Funakoshi *et al.*, 2000). We found one case with G to A in intron 1, and another case with C to G in exon 3, without any change in amino acid (Thr). The polymorphisms of the promoter region (between −351 and +176) were also examined, and no polymorphisms other than −81A to G and −128G to T were detected. Those designated as −333G/T and −286A/G by Okubo *et al.* (2002) were identical to −128G/T and −81A/G in the present study, respectively. No association of these polymorphisms (−128G/T and −81A/G) with alcohol dependence was observed (Okubo *et al.*, 2002). One possible explanation for the differences between the study by Okubo *et al.* (2002) and our study is that Okubo *et al.* (2002) did not exclude subjects with inactive ALDH2. Inactive ALDH2 (2*1/2*2 and 2*2/2*2) is a strong negative risk factor for alcohol dependence (Higuchi *et al.* 1995). Tachikawa *et al.* (2000) reported an association of the 201A allele (201A/G is identical to −81A/G in the present study) of the CCK-AR gene with schizophrenia. Given the potential differences between alcohol dependence and other psychiatric disorders, our results do not completely contradict their findings.

We recently reported that functional comparison of the A and G variants of the −81 A/G polymorphism by luciferase assay demonstrated a slight decrease in the G variant, but no

significant difference (Takata *et al.*, 2002). However, we used STC-1 (Rindi *et al.*, 1990), established from a transgenic mouse expressing a viral oncogene under the control of the insulin promoter, because no human-derived cell line expressing CCK-AR was available. Further studies employing various experimental conditions are needed before conclusions can be drawn regarding the effect of this polymorphism on expression of the CCK-AR gene.

A recent report mapped the CCK-AR gene to chromosome 4 (4p15.2–15.1), in the vicinity of the dopamine D5 receptor gene (4p16.1–15.1) (Beischlag *et al.*, 1995). The dopamine D5 receptor binds dopamine with a 10-fold greater affinity than that of dopamine receptor 1. The dopamine D5 receptor protein is also localized in the prefrontal cortex. Thus, alterations in the CCK-AR gene may lead to some modification of dopamine release, and alteration of dopaminergic neurotransmission may be involved in alcohol misuse (Crawley, 1991; Marshall *et al.*, 1991; Woodruff *et al.*, 1991; Kalivas, 1993; Ladurelle *et al.*, 1994; Hamilton and Freeman, 1995; Self and Nestler, 1995; Wank, 1995).

In summary, the CCK-AR gene –81A/G polymorphism was found to be associated with alcohol dependence, and the –81G allele of the CCK-AR gene to be possibly associated with intractable alcohol dependence.

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PRELIMINARY REPORT

Association of a Polymorphism of the Matrix Metalloproteinase-9 Gene With Bone Mineral Density in Japanese Men

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Matrix metalloproteinase-9 (MMP-9) is implicated in bone remodeling. A −1562C→T polymorphism in the promoter of the MMP-9 gene (*MMP9*) has been shown to influence gene transcription. The possible relation of this polymorphism to bone mineral density (BMD) was examined in 1,114 Japanese men and 1,087 women. BMD for the total body, lumbar spine, femoral neck, trochanter, or Ward's triangle was significantly lower in the combined group of men with the *CT* or *TT* genotypes or in men with the *CT* genotype than in those with the *CC* genotype. No significant differences in BMD among *MMP9* genotypes were observed in premenopausal or postmenopausal women. The −1562C→T polymorphism of *MMP9* was thus associated with BMD in Japanese men.

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MATRIX metalloproteinase-9 (MMP-9) is produced by osteoclasts in human bone and is implicated both in bone resorption,[1-3] as well as in bone formation.[4] A C→T polymorphism at position −1562 in the promoter of the MMP-9 gene (*MMP9*) has been shown to affect transcriptional activity, with the T allele being associated with increased gene transcription.[5] We have now examined whether this polymorphism is associated with bone mineral density (BMD) in a population-based study.

MATERIALS AND METHODS

The National Institute for Longevity Sciences–Longitudinal Study of Aging is a population-based prospective cohort study of aging and age-related diseases.[6] We examined the possible association of BMD at various sites with the −1562C→T polymorphism of *MMP9* in 1,114 Japanese men and 1087 women. The study protocol was approved by the Committee on the Ethics of Human Research of the National Institute for Longevity Sciences, and written informed consent was obtained from each subject. BMD for the total body, lumbar spine (L2 to L4), 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. The polymorphic region of *MMP9* was amplified by the polymerase chain reaction with allele-specific sense primers labeled at the 5′ end with either fluorescein isothiocyanate (5′-CCGAGTAGCTGGTATTATAGGXAT-3′) or Texas red (5′-CGAGTAGCTGGTATTATAGGXGT-3′) and with an antisense primer labeled at the 5′ end with biotin (5′-AAACCAGCCTGGT-CAACGTA-3′). The reaction mixtures (25 μL) contained 20 ng of DNA, 5 pmol of each primer, 0.2 mmol/L of each deoxynucleoside triphosphate, 4.5 mmol/L $MgCl_2$, and 1 U of Taq DNA polymerase in buffer. The amplification protocol comprised initial denaturation at 95°C for 5 minutes, 35 cycles of denaturation at 95°C for 30 seconds, annealing at 66.5°C for 30 seconds, extension at 68°C for 30 seconds, and a final extension at 68°C for 2 minutes. 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 from each well were transferred to the wells of a 96-well plate containing 0.01 mol/L NaOH and then measured for fluorescence with a microplate reader.

Quantitative data were compared among 3 groups by 1-way analysis of variance and the Tukey-Kramer post hoc test, and between 2 groups by the unpaired Student's *t* test. BMD values were analyzed with

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Table 1. BMD and Other Characteristics of Men (n = 1,114) or of Premenopausal (n = 279) or Postmenopausal (n = 808) Women According to the −1562C→T Genotype of *MMP9*

Characteristic	*CC*	*CT*	*TT*	*CT* + *TT*
Men				
No. (%)	794 (71.3)	280 (25.1)	40 (3.6)	320 (28.7)
Age (yr)	59.0 ± 0.4	59.9 ± 0.7	58.7 ± 1.7	59.7 ± 0.6
BMI (kg/m²)	22.9 ± 0.1	22.8 ± 0.2	23.1 ± 0.4	22.9 ± 0.2
Fracture (%)	201 (25.3)	76 (27.1)	11 (27.5)	87 (27.2)
BMD values (g/cm²)				
Total body	1.090 ± 0.003	1.076 ± 0.006	1.081 ± 0.015	1.077 ± 0.005*
L2-L4	0.988 ± 0.006	0.965 ± 0.010	0.981 ± 0.026	0.967 ± 0.009*
Femoral neck	0.758 ± 0.004	0.739 ± 0.006*	0.736 ± 0.017	0.739 ± 0.006†
Trochanter	0.673 ± 0.004	0.655 ± 0.006*	0.659 ± 0.017	0.655 ± 0.006*
Ward's triangle	0.559 ± 0.004	0.534 ± 0.007*	0.532 ± 0.020	0.534 ± 0.007‡
Premenopausal women				
No. (%)	200 (71.7)	70 (25.1)	9 (3.2)	79 (28.3)
Age (yr)	46.2 ± 0.3	45.6 ± 0.5	49.9 ± 1.5§	46.1 ± 0.5
BMI (kg/m²)	22.8 ± 0.2	22.8 ± 0.4	22.7 ± 1.1	22.8 ± 0.4
Fracture (%)	23 (11.5)	8 (11.4)	3 (33.3)	11 (13.9)
BMD values (g/cm²)				
Total body	1.091 ± 0.006	1.102 ± 0.010	1.088 ± 0.028	1.100 ± 0.009
L2-L4	1.019 ± 0.009	1.035 ± 0.014	1.031 ± 0.041	1.035 ± 0.014
Femoral neck	0.770 ± 0.007	0.775 ± 0.016	0.793 ± 0.033	0.777 ± 0.011
Trochanter	0.654 ± 0.006	0.664 ± 0.011	0.689 ± 0.030	0.667 ± 0.010
Ward's triangle	0.659 ± 0.009	0.654 ± 0.015	0.693 ± 0.042	0.658 ± 0.014
Postmenopausal women				
No. (%)	563 (69.7)	214 (26.5)	31 (3.8)	245 (30.3)
Age (yr)	63.9 ± 0.4	64.1 ± 0.6	65.0 ± 1.5	64.2 ± 0.5
BMI (kg/m²)	23.0 ± 0.1	22.8 ± 0.2	23.3 ± 0.6	22.9 ± 0.2
Fracture (%)	114 (20.2)	45 (21.0)	8 (25.8)	53 (21.6)
BMD values (g/cm²)				
Total body	0.920 ± 0.004	0.915 ± 0.006	0.914 ± 0.016	0.915 ± 0.006
L2-L4	0.808 ± 0.006	0.806 ± 0.009	0.841 ± 0.025	0.810 ± 0.009
Femoral neck	0.645 ± 0.004	0.642 ± 0.006	0.637 ± 0.016	0.641 ± 0.006
Trochanter	0.540 ± 0.004	0.538 ± 0.006	0.533 ± 0.016	0.537 ± 0.006
Ward's triangle	0.452 ± 0.005	0.451 ± 0.008	0.461 ± 0.022	0.452 ± 0.008

NOTE. Data are means ± SE. BMD values are adjusted for age.
*$P < .05$, †$P < .01$, ‡$P < .005$ *v CC*.
§$P < .05$ *v CC* or *CT*.

adjustment for age by the least squares method in a general linear model. A P value < .05 was considered statistically significant.

RESULTS

Age, body mass index (BMI), and the prevalence of nontraumatic fractures did not differ among −1562C→T genotypes in men or in premenopausal or postmenopausal women (Table 1). We compared BMD values among the 3 genotypes (*CC, CT,* and *TT*), as well as between 2 groups of genotypes in dominant (*CC* and *CT* + *TT*) and recessive (*CC* + *CT* and *TT*) genetic models to examine the effect of the *T* allele on BMD. BMD for the total body, lumbar spine, femoral neck, trochanter, or Ward's triangle was significantly lower in the combined group of men with the *CT* or *TT* genotypes or in men with the *CT* genotype than in those with the *CC* genotype (Table 1). The differences in BMD between men with the *CC* genotype and those with either the *CT* or *TT* genotypes (expressed as a percentage of the corresponding larger value) were 1.5% for the total body, 2.2% for the lumbar spine, 2.8% for the femoral neck, 2.7% for the trochanter, and 5.2% for Ward's triangle. For premenopausal or postmenopausal women, BMD did not differ among −1562C→T genotypes (Table 1).

DISCUSSION

We previously showed that the −1607G→GG polymorphism of *MMP1* was associated with BMD at the radius in postmenopausal women,[6] with the *GG* genotype, which exhibits an increased transcriptional activity,[7] representing a risk factor for reduced BMD. The T allele of the −1562C→T polymorphism in the promoter of *MMP9* also exhibits higher transcriptional activity than does the *C* allele.[5] A 9-bp sequence (−1567 to −1559) containing the −1562C→T site has been suggested to function as an important regulatory element by serving as a binding site for a transcriptional repressor protein. In addition, the serum concentration of MMP-9 was shown to

be higher in individuals with the *TT* genotype than in those with the *CC* or *CT* genotypes.[5] We have now shown that the −1562C→T polymorphism of *MMP9* was associated with BMD at various sites in Japanese men, with the *T* allele being related to reduced bone mass. Given that MMP-9 degrades collagen in the bone matrix, an increased activity of this protease might be expected to result in reduced bone mass. Our results are thus consistent with the previous observations that the *T* allele of *MMP9* exhibits higher transcriptional activity and is associated with a higher serum concentration of the encoded protein.[5]

Given that BMD values for the total body, lumbar spine, femoral neck, trochanter, and Ward's triangle in men with the *TT* genotype were similar to those in men with the *CT* genotype, the *T* allele may exert a dominant effect on BMD. The lack of statistical significance for differences in BMD between the *CC* and *TT* genotypes may be attributable to the small number of subjects with the *TT* genotype (n = 40), compared with the number of those with the *CT* genotype (n = 280). This polymorphism was associated with BMD in men but not in women. The reason for this gender difference remains unclear, but differences in the concentrations of estrogen and other sex hormones between men and women might be contributing factors. Although it is possible that the −1562C→T polymorphism of *MMP9* is in linkage disequilibrium with polymorphisms of other nearby genes that are actually responsible for reduced BMD, our present results suggest that this polymorphism of *MMP9* is associated with BMD in Japanese men.

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Inactive Aldehyde Dehydrogenase-2 Increased the Risk of Pancreatic Cancer Among Smokers in a Japanese Male Population

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Objectives: Most of the acetaldehyde, a recognized animal carcinogen, generated during alcohol metabolism is eliminated by liver mitochondrial aldehyde dehydrogenase 2 (ALDH2). More than 40% of Japanese have the inactive form of ALDH2, and inactive ALDH2 is a risk factor for multiple cancers of the esophagus as well as head and neck cancer. Possible associations between pancreatic cancer and ALDH2 gene polymorphism, in conjunction with smoking and/or drinking habits, were examined in a Japanese population.

Methods: We investigated 114 patients (70 male and 44 female) with pancreatic cancer and compared them with 2070 control subjects (1050 male and 1020 female). The drinking (5 g ethanol consumption/d) and/or smoking habits as well as ALDH2 gene polymorphism were examined.

Results: In male subjects, the frequency of the active form of ALDH2 (2*1/2*1) was lower in pancreatic cancer patients than in control subjects ($P = 0.018$). The frequency of subjects with both smoking and drinking habits was significantly higher in pancreatic cancer patients than in control subjects having ALDH2*1/2*1 and ALDH2*1/2*2. The frequency of smoking habit alone was significantly higher in pancreatic cancer patients compared with control subjects having inactive ALDH2. Drinking habit had no relation to pancreatic cancer. In female subjects, neither habit had a relation to pancreatic cancer.

Conclusions: Smoking habit did increase the risk of pancreatic cancer, and this risk was further enhanced in subjects with inactive ALDH2 in a male population but not in a female population. There was no relationship between drinking habit and pancreatic cancer in either sex population.

Key Words: pancreatic cancer, alcohol, smoking, ALDH2, genotype

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In Japan, pancreatic cancer ranks as the fifth most common cause of cancer death, and the 5-year survival rate of its victims is less than 10%.[1] Smoking is a well-documented risk factor for the development of pancreatic adenocarcinoma.[2] In contrast, alcohol intake has not been firmly established as causally related or unrelated to pancreatic cancer.[3,4] Heavy alcohol intake may cause chronic pancreatitis. Alcoholic pancreatitis, which accounts for 55.5% of pancreatitis cases, is the most common type in Japanese men (68.5%).[5] Chronic pancreatitis has been indicated as a risk factor for pancreatic cancer.[6–9]

In the body, alcohol is processed as follows. Orally ingested ethanol is metabolized by alcohol dehydrogenase, and the first metabolite is acetaldehyde. Most of the acetaldehyde generated during alcohol metabolism is eliminated by liver mitochondrial aldehyde dehydrogenase 2 (ALDH2) by converting the acetaldehyde into acetic acid. The Japanese population is deficient in ALDH2 because of the high frequency of a mutant allele in the ALDH2 gene (ALDH2*2). The ALDH2*2 allele encodes a Glu-to-Lys amino acid substitution at the 14th and last codon. More than 40% of Japanese have the inactive form of ALDH2, encoded as either heterozygous ALDH2*1/2*2 or homozygous ALDH2*2,[10] while the majority of whites possess the active form of ALDH2 (2*1/2*1).

Acetaldehyde is a recognized animal carcinogen.[11] A recent report[12] showed that inactive ALDH2 is a risk factor for multiple carcinomas of the esophagus in alcoholics and that acetaldehyde appears to play a critical role in field cancerization. More recently, there has been an association between ALDH2 gene polymorphisms and cancers of the head and neck.[13]

In this study, we investigated 114 Japanese patients with pancreatic cancer to determine whether it is associated with ALDH2 gene polymorphism, particularly in conjunction with smoking and/or drinking habits.

MATERIALS AND METHODS

Subjects

This study was approved by the Ethics Committees of the National Kyushu Cancer Center, of the National Institute of Longevity Sciences (NILS), and of the Tokyo Metropolitan Institute of Gerontology. Written informed consent was obtained from each subject.