文　献

1) Howland, J., Peterson, E. W., Levin, W. C. et al.: Fear of falling among the community-dwelling elderly. J. Aging Health 5: 229-243, 1993.
2) 金　憙経，吉田英世，鈴木隆雄ほか：高齢者の転倒関連恐怖感と身体機能—転倒外来受診者について—．日老医会誌 38: 805-811, 2001.
3) 鈴木みずえ，金森雅夫，山田紀代美：在宅高齢者の転倒恐怖感(fear of falling)とその関連要因に関する研究．老年精医誌 10: 685-695, 1999.
4) Howland, J., Lachman, M. E., Peterson, E. W: et al.: Covariates of fear of falling and associated activity curtailment. Gerontologist 38: 549-555, 1998.
5) Cumming, R. G., Salkeld, G., Thomas, M. et al.: Prospective study of the impact of fear of falling on activities of daily living, SF-36 scores, and nursing home admission. J. Gerontol. A Biol. Sci. Med. Sci. 55: M299-M305, 2000.
6) Shimokata, H., Ando, F. and Niino, N.: A new comprehensive study on aging—the National Institute for Longevity Sciences, Longitudinal Study of Aging (NILS-LSA). J. Epidemiol. 10: S1-S9, 2000.
7) 古谷野亘，柴田　博，中里克治ほか：地域老人における活動能力の測定—老研式活動能力指標の開発．日公衛誌 34: 109-114, 1987.
8) Niino, N., Imaizumi, T. and Kawakami, N.: Japanese translation of the Geriatric Depression Scale. Clin. Gerontol. 10: 85-87, 1991.
9) 厚生労働省監修：平成13年版厚生労働白書—生涯にわたり個人の自立を支援する厚生労働行政—，ぎょうせい，東京，2001.

日本未病システム学会雑誌　10(1)：100-102，2004

研究報告●17

中高年者の口腔所見に関する研究

譽田　英喜[1)]　新井　康司[2)]　角　保徳[1)]　安藤富士子[3)]
新野　直明[4)]　下方　浩史[3)]

1．背景および目的

加齢に伴い身体には様々な変化が生じる。特に口腔の変化は，高齢者の栄養摂取に大きな影響を与えるだけでなく，脳梗塞[1)]，冠動脈疾患[2)]，糖尿病[3)]などの生活習慣病，老年病の要因となる。しかしわが国においては，一般地域住民を対象とした大規模な口腔調査はほとんど実施されていない。本研究の目的は，地域住民を対象に，中高年者の保有歯数，歯周組織の状態および舌苔の量の実態を年代別，性別に明らかにすることである。

2．対象と方法

対象は，国立長寿医療研究センター（現・国立長寿医療センター）疫学研究部が行っている「老化に関する長期縦断疫学調査」の第三次調査のうち，2002年5月から2003年2月までに参加した40～84歳の910名（男性461名，女性449名）である。

口腔診査は歯科診療室にて歯科医師が行った。

1．保有歯数

視診にて保有歯数を調べた。

2．歯周組織

WHO基準のCPI部分診査法[4)]に準じて，対象6歯に関し，0：健全歯周組織，1：診査時出血，2：歯石付着，不良辺縁をもつ修復物がある，3：歯周ポケット4～6 mm，4：歯周ポケット6 mm以上と評価し，対象6歯において最も悪い所見をCPIの評価値とした。また，対象6歯がすべてない者に関しては評価を5とした。

表1　保有歯数の年代・性別分布（本）

年代	男性	女性
40歳代	27.6±0.6	27.5±0.6
50歳代	25.9±0.6	25.6±0.6
60歳代	22.2±0.5	22.5±0.5
70歳代	14.2±0.5	14.9±0.6
80歳代	12.0±1.4	10.5±1.8

（平均値±標準誤差）

男女ともに年代が高い者ほど保有歯数が減っていた（trend $p<0.001$）。

t検定による各年代における保有歯数の性差はすべて有意でなかった。

3．舌苔

Miyazakiらの方法[5)]に準じて，0：舌苔無，1：舌苔分布が舌背の1/3未満，2：同1/3以上2/3未満，3：同2/3以上の4段階に分類評価した。

参加者を40歳から10歳ごとの年代別，性別に分け，比較検討を行った。統計解析にはSAS release 8.2を使用した。保有歯数の年代による変化については一般線形モデルによるトレンド検定を，性差に関してはt検定を行った。歯周組織の状態および舌苔の量に関しては，Cochran-Mantel-Haenszel法にて年代によるトレンド検定および年齢を調整しての性差の検定を行い，$p<0.05$を統計学的有意とした。本研究は国立療養所中部病院（現・国立長寿医療センター）倫理委員会の了承の下に，調査への参加の文書による同意の得られた者を対象として行われた。

1）国立長寿医療センター病院先端医療部口腔機能再建科　2）国立長寿医療センター研究所運動器疾患研究部　3）国立長寿医療センター研究所疫学研究部　4）桜美林大学大学院老年学

表2　歯周組織評価(CPI)の年代・性別分布(%)

CPI	男性					女性				
	40歳代	50歳代	60歳代	70歳代	80歳代	40歳代	50歳代	60歳代	70歳代	80歳代
0	10.1	2.0	1.6	1.7	0.0	19.1	3.4	2.5	0.0	0.0
1	1.0	0.0	0.8	0.0	0.0	1.8	0.0	0.0	1.1	0.0
2	40.4	26.6	25.2	14.4	28.6	54.6	48.7	35.0	22.8	33.3
3	31.3	37.2	33.1	24.6	4.8	16.4	27.4	30.0	41.3	22.2
4	17.2	31.9	33.9	35.6	38.1	8.2	18.8	27.5	19.6	0.0
5	0.0	2.3	5.4	23.7	28.5	0.0	1.7	5.0	15.2	44.5

男女とも年代が高い者ほど歯周組織の状態が悪かった($p<0.001$)。
また，年代を調整してのCPIは，男性の方が女性よりも悪かった($p<0.001$)。
(Cochran-Mantel-Haenszel検定)

表3　舌苔の年代・性別分布(%)

舌苔	男性					女性				
	40歳代	50歳代	60歳代	70歳代	80歳代	40歳代	50歳代	60歳代	70歳代	80歳代
0	67.7	50.0	47.7	47.1	42.9	82.7	69.2	72.5	60.2	55.6
1	23.2	41.5	35.2	32.8	33.3	14.6	20.5	19.2	31.2	33.3
2	8.1	8.5	16.4	18.5	23.8	2.7	8.6	7.5	8.6	11.1
3	1.0	0.0	0.7	1.6	0.0	0.0	1.7	0.8	0.0	0.0

男女とも年代が高い者ほど舌苔の量が多かった($p<0.001$)。
また，年代を調整しての舌苔の量は，男性の方が女性よりも多かった($p<0.001$)。
(Cochran-Mantel-Haenszel検定)

3．結果

平均保有歯数は，40歳代では男性が27.6本，女性が27.5本であったが，80歳代ではそれぞれ12.0本，10.5本と，男女とも年代の上昇に伴い減少していた($p<0.001$)。また各年代において平均保有歯数の性差は認められなかった(表1)。

歯周組織に関しては，男女とも年代が高い者ほど状態が悪かった($p<0.001$)。また，年代を調整して比較したところ，女性よりも男性の方が歯周組織の状態が悪かった($p<0.001$)(表2)。

舌苔に関しては，男女とも年代が高い者ほど認められる者が多かった($p<0.001$)。また，年代を調整しての男女を比較したところ，男性は女性に比べ舌苔の量が多かった(表3)。

4．考察

ヒトの永久歯は親知らずを含め32本が標準である。平成11年度の歯科疾患実態調査によれば，平均保有歯数は40，50，60，70，80歳代ではそれぞれ27.5，24.3，22.2，14.3，8.2本であった[6]。今回のわれわれの結果は上記調査と同程度であり，本研究の対象者は日本における標準的な集団と考える。

歯周病は齲蝕と並んで歯牙喪失の大きな原因となっており，歯牙は歯周組織が良い状態で残っていることが重要である。本調査では年代の上昇に伴い歯周組織の状態が悪化しており，また男性の方が女性に比べ歯周組織の状態が悪かったが，この結果は他の報告と同じであった[7,8]。

歯牙喪失は60歳代で顕著であるが，喪失の原因である歯周病は40歳代から50歳代にかけて既に悪化が認められていた。このことから，歯周組織の状態が悪くなる前の40歳代における口腔衛生管理が重要であると考えられる。

舌苔は，口腔粘膜の上皮細胞や口腔微生物などから構成され，口臭の原因になるといわれている[5]。年代上昇に伴い舌苔の認められる者が多くなってきており，今後舌

清掃指導も必要になると考えられる。

今後，さらに歯牙喪失や口腔衛生の状態と老化や老年病との関わりを明らかにして，疾病予防の手がかりとしていきたい。

5 結論

年代上昇に伴って保有歯数の減少，歯周組織の悪化，また舌苔の増加が認められ，加齢による口腔の変化が示唆された。

文　献

1）Joshipura, K. J., Hung, H. C., Rimm, E. B. et al.：Periodontal disease, tooth loss, and incidence of ischemic stroke. Stroke 34：47-52, 2003.

2）DeStefano, F., Anda, R. F., Kahn, H. S. et al.：Dental disease and risk of coronary heart disease and mortality. BMJ 306：688-691, 1993.

3）Miller, L. S., Manwell, M. A., Newbold, D. et al.：The relationship between reduction in periodontal inflammation and diabetes control：a report of 9 cases. J. Periodontol. 63：843-848, 1992.

4）Ainamo, J., Barmes, D., Beagrie, G. et al.：Development of the World Health Organization (WHO) community periodontal index of treatment needs (CPITN). Int. Dent. J. 32：281-291, 1982.

5）Miyazaki, H., Sakao, S., Katoh, Y. et al.：Correlation between volatile sulphur compounds and certain oral health measurements in the general population. J. Periodontol. 66：679-684, 1995.

6）厚生労働省医政局歯科保健課編：平成11年歯科疾患実態調査報告，口腔保健協会，東京，2001.

7）Ajwani, S., Tervonen, T., Narhi, T. O. et al.：Periodontal health status and treatment needs among the elderly. Spec. Care Dentist. 21：98-103, 2001.

8）Grossi, S. G., Zambon, J. J., Ho, A. W. et al.：Assessment of risk for periodontal disease. I. Risk indicators for attachment loss. J. Periodontol. 65：260-267, 1994.

ELSEVIER

Cholecystokinin A Receptor Gene Promoter Polymorphism and Intelligence

HIROSHI SHIMOKATA, MD, PHD, FUJIKO ANDO, MD, PHD, NAOAKIRA NIINO, MD, PHD, KYOKO MIYASAKA, MD, PHD, AND AKIHIRO FUNAKOSHI, MD, PHD

PURPOSE: To study the association between Cholecystokinin A receptor (CCKAR) genotypes and intelligence in community-living men and women.
METHOD: Subjects were 2251 community-dwelling Japanese men and women aged 40 to 79 years. The CCKAR gene promoter polymorphisms A-81G and G-128T were determined. Intelligence was assessed by Japanese Wechsler Adult Intelligence Scales – Revised Short Forms (JWAIS-R SF). The difference in intelligence between wild type and mutation was tested.
RESULTS: There were no subjects with *AA/GT*, *AA/TT*, or *AG/TT* genotypic combinations. Both A-81G and G-128T genotypes were related to intelligence quotient (IQ) estimated by JWAIS-R SF. The mean and SE of IQ levels of subjects with the wild-type allele and the mutation allele at nucleotide -128 were 103.4 ± 0.3 and 101.6 ± 0.6, respectively. There was a significant difference in IQ for G-128T ($p = 0.008$). The difference in IQ for A-81G was also significant ($p = 0.011$). The IQ level was 103.6 ± 0.4 in the subjects with the wild-type allele and 102.0 ± 0.5 in the subjects with the mutation. Differences in IQ levels by haplotypes for combinations of A-81G/G-128T were examined. IQ significantly decreased with an increasing number of mutation alleles ($p = 0.018$).
CONCLUSION: There were statistically significant differences in IQ for CCKAR gene promoter polymorphisms A-81G and G-128T in community-living Japanese.
Ann Epidemiol 2005;15:196–201.

KEY WORDS: Cholecystokinin, Intelligence, Genotype, Epidemiology.

INTRODUCTION

It is suspected that various genes influence intelligence, but the association between gene polymorphism and intelligence is still unclear. Cholecystokinin (CCK) is one of the major physiologic substances of gallbladder contraction and pancreatic enzyme secretion. CCK also plays an important role in the central nervous system (CNS) by interacting with dopamine and other neurotransmitters (1). CCK receptors have been classified into two subtypes, CCK type-A receptor (CCKAR) and type-B receptor (CCKBR). CCKAR has been found in the CNS (2). Associations with feeding disorders (3), anxiety (4), and schizophrenia (5) have been reported. It was also reported that learning and memory functions were impaired in CCKAR gene-knockout (OLETF) rats (6, 7). The CCKAR gene may be related to intelligence in humans. We examined the association between CCKAR gene promoter polymorphisms and intelligence in a group of 2251 community-dwelling Japanese men and women.

METHODS

Subject Selection

The subjects in this study were participants in the National Institute for Longevity Sciences – Longitudinal Study of Aging (NILS-LSA) (8). The NILS-LSA started in November 1997. The first phase of examinations was finished by the end of March 2000, and followed-up every 2 years. Participants in the NILS-LSA were independent residents in Obu city and Higashiura town in Aichi prefecture, central Japan. Data on all residents in the area are maintained in a Resident Registration System by local governments. Residents aged 40 to 79 years old were selected using Resident Registration. Samples of 7790 males and females were selected by age and gender stratified random sampling and invited to an explanatory meeting by mail. The number of replies was 3434. Of these, 881 refused to attend the meeting, 2553 agreed to attend, and 2513 actually attended. After the meeting, 2267 participated in the first phase examination. At the meeting, the procedures

From the Department of Epidemiology, National Institute for Longevity Sciences, Obu Aichi, Japan (H.S., F.A., N.N.); Department of Clinical Physiology, Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan (K.M.); and Department of Gastroenterology, National Kyushu Cancer Center, Fukuoka, Japan (A.F.).
Address correspondence to: Hiroshi Shimokata, M.D., Ph.D., Department of Epidemiology, National Institute for Longevity Sciences, 36-3 Gengo, Morioka-cho, Obu Aichi 474-8522, Japan. Tel.: +81-562-46-2311, ext. 815; Fax: +81-562-44-6593. E-mail: hiroshi@nils.go.jp
This work was supported in part by Research Grants for Longevity Sciences (12C-01) from the Ministry of Health and Welfare of Japan to Drs. H. Shimokata and A. Funakoshi.
Received June 6, 2004; accepted June 14, 2004.

360 Park Avenue South, New York, NY 10010
1047-2797/05/$–see front matter
doi:10.1016/j.annepidem.2004.06.007

Selected Abbreviations and Acronyms

BMI = body mass index
CCK = cholecystokinin
CCKAR = cholecystokinin A receptor
CNS = central nervous system
DNA = deoxyribonucleic acid
GLM = general linear model
IQ = intelligence quotient
JWAIS-R-SF = Japanese Wechsler Adult Intelligence Scales – Revised Short Forms
NILS-LSA = National Institute for Longevity Sciences – Longitudinal Study of Aging
PCR-RFLP = polymerase chain reaction – restriction fragment length polymorphism
OLETF = Otsuka Long-Evans Tokushima Fatty
SE = standard error
WAIS-R = Wechsler Adult Intelligence Scales – Revised

for each examination and follow-up schedule were fully explained. Written informed consent to participate in all procedures was obtained from each subject. All persons in the Resident Registration list had Japanese nationality, and there were no persons who had a foreign name among the subjects. The subjects in this study were supposed to be ethnically homogenous Japanese.

Among the 2267 participants in the first phase examination, 2251 men and women were evaluated for CCKAR genotypes and intelligence. These subjects were analyzed for cross-sectional associations between genotype and intelligence. The number of the subjects by gender and age was almost equal (Table 1). The mean and standard deviation for age was 59.2 ± 10.9 years. Among the subjects, 26.7% had an educational background of college or greater. The Ethical Committee of Chubu National Hospital approved all procedures of the NILS-LSA.

Evaluation of Intelligence and Other Variables

The Wechsler Adult Intelligence Scales – Revised (WAIS-R) is one of the most popular tools used to assess intelligence (9). A Japanese version of the WAIS-R (JWAIS-R) has been developed and is widely used in Japan (10). In this study, intelligence was assessed by the Japanese Wechsler Adult Intelligence Scales - Revised - Short Forms (JWAIS-R-SF) (11). The JWAIS-R-SF consists of the following four subtests: Information, Similarities, Picture Completion, and Digit Symbol. Scaled scores of subtests were used in the analysis. The intelligence quotient (IQ) was estimated from the combination of these four subtests. Psychologists conducted the interviews and JWAIS-R-SF tests. Height and weight were measured while wearing lightweight clothes, and body mass index (BMI) was calculated as weight (kg) divided by height (m) squared. Life-style and medical history including annual income, education, and smoking status were checked by questionnaires. The questionnaires were checked by a physician at the medical examination. All drugs used during the previous 2 years were to be documented by participants; the physician confirmed them at an interview and coded the drugs used during the last 2 weeks. Among the 2251 subjects in the study, 213 had used drugs acting on the CNS, that is, hypnotic sedative agents, antianxiety agents, antiepileptic agents, stimulant drugs, antihypnotic drugs, anti-Parkinson drugs, and anti-psychotic drugs during the previous 2 weeks. The IQ was less than 70 in 11 subjects, and only one of them used drugs acting on the CNS.

TABLE 1. Distribution of the subjects by gender and age

	Age (years)				
Gender	40–49	50–59	60–69	70–79	Total
Males	291	282	281	280	1134
Females	278	278	283	278	1117
Total	569	560	564	558	2251

CCKAR Genotype Analysis

Genomic DNA was extracted from peripheral blood lymphocytes by a standard procedure. A mismatch PCR-RFLP method was used to analyze polymorphisms in the upstream region of the CCKAR gene [GenBank Accession No. U23427 (5)]. One pair of primers, sense primer = 5′-GCATATGTACACATGTGTGTAAAAAGCAGCCAGAC-3′, anti-sense primer = 5′-GCCCTTTCCTGGGCCAGACT-3) was designed to amplify a 103-base pair product, digested with restriction enzyme Hinf I, and analyzed by 3% agarose gel electrophoresis. Two sequence changes were detected: a G to T change at nucleotide -128, and an A to G change at nucleotide -81 (12).

Statistical Analysis

All values were expressed as the mean ± SE, if not specified. Both polymorphisms at nucleotides -128 and -81 were divided into two groups; as wild-type and mutation. Hetero groups were classified as mutation. The difference between wild-type and mutation groups was tested by the *t*-test for continuous variables and the 2 × 2 chi-square test for categorical variables. The difference in IQ and JWAIS-R subtests score by genotype was also tested by the *t*-test excluding subjects who had used drugs acting on the CNS or subjects with IQ less than 70. The trend among the three groups was tested by the general linear model (GLM) and the probability for trend (p for trend) was shown. Statistical analyses were performed using the SAS system (SAS Institute Inc., Cary, NC). All p-values were two-tailed.

AEP Vol. 15, No. 3
March 2005: 196–201

RESULTS

Distribution of CCKAR Promoter Genotypes

The distributions of CCKAR promoter single nucleotide polymorphisms A-81G and G-128T were both in Hardy-Weinberg equilibrium. The distribution of genotype combination was examined (Table 2). These polymorphisms were in linkage disequilibrium. There were no subjects with *AA/GT*, *AA/TT*, or *AG/TT* genotypic combinations. Thus, subjects with a mutation at -128 always had a mutation at -81.

Background Characteristics and CCKAR Genotype

Figure 1 shows the IQ distribution. The distribution was slightly skewed to the left (lower IQ) and close to a normal distribution. The mean value of the IQ of the all subjects was 103.0, and the median was also 103. The difference between the mean and median was very small. The lowest IQ was 43 and the highest IQ was 142 among the subjects. The number of subjects with IQ less than 70 was 11, and those with IQ 135 or over was 13. Background characteristics were compared by CCKAR G-128T and A-81G genotypes (Table 3). Age, body weight, body mass index, annual income, education, and smoking status did not differ between wild-type (GG) and mutation (GT or TT) for the CCKAR G-128T genotype. These variables also did not differ for the CCKAR A-81G genotype except for education status. Education status in the wild-type (AA) group was significantly higher than that in the mutation-type (AG or GG) group ($p = 0.009$). The IQ was significantly different by education status ($p < 0.001$). The IQ for the low education group was 100.3 ± 0.3 and that for the high education group was 110.6 ± 0.5.

Intelligence and CCKAR Genotype

The IQ levels in subjects with wild-type and mutation alleles at nucleotide -128 were 103.4 ± 0.3 and 101.6 ± 0.6, respectively. There was a significant difference in IQ for the G-128T genotype ($p = 0.008$). The score of Digit Symbol was lower in subjects with a mutation ($p = 0.003$). There was no difference in the scores of Information, Picture Completion, and Similarities subtests for polymorphism G-128T. The IQ level was 103.6 ± 0.4 in subjects with wild-type (AA) and 102.0 ± 0.5 in subjects with mutation (AG or GG) at nucleotide -81. The difference in IQ for the A-81G polymorphism was significant ($p = 0.011$). The Picture Completion and Digit Symbol subtest scores were significantly lower in subjects with the mutation ($p = 0.043$ and $p = 0.008$, respectively). The Similarities subtest score was marginally lower for a mutation at nucleotide -81 ($p = 0.051$).

In the low education group, IQ was 100.5 ± 0.4 in the -128 wild-type group and 99.5 ± 0.6 in the -128 mutation-type group. There was no significant difference in IQ between the wild- and mutation-type of G-128T genotype. However, the IQ for the -81 wild-type group was 100.8 ± 0.4, which was significantly higher than that for the mutation group (99.4 ± 0.4) ($p = 0.038$). In the high education group, the IQ was 111.5 ± 0.6 in the -128 wild-type group and 107.9 ± 1.1 in the -128 mutation-type group. There was a significant difference between the wild and mutation groups ($p = 0.004$). The IQ in the -81 wild-type group (111.1 ± 0.7) did not differ from that in the mutation group (109.8 ± 0.9).

Intelligence was compared excluding subjects who had used drugs acting on the CNS and subjects with IQ less than 70 (Table 4). The number of excluded subjects was 223. Differences in IQ between in the wild-type and mutation groups were still significant both for A-81G and G-128T

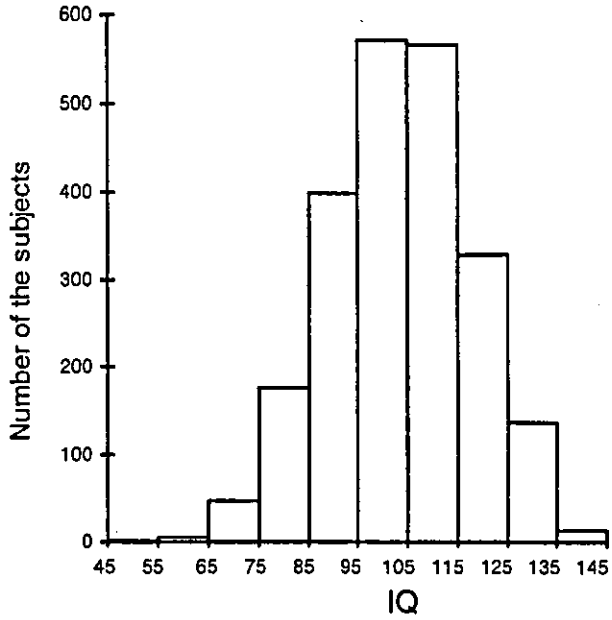

FIGURE 1. Distribution of IQ levels in the subjects.

TABLE 2. Distribution of CCKAR G-81T and A-128G genotypes

CCKAR G-128T	CCKAR A-81G AA	AG	GG	Total
GG	1317 (58.5%)	307 (13.6%)	26 (1.2%)	1650 (73.3%)
GT	0 (0.0%)	491 (21.8%)	61 (2.7%)	552 (24.5%)
TT	0 (0.0%)	0 (0.0%)	49 (2.2%)	49 (2.2%)
Total	1317 (58.5%)	798 (35.5%)	136 (6.0%)	2251 (100.0%)

TABLE 3. Comparison of variables between wild-type and mutation alleles in CCKAR G-81T and A-128G genotypes

	CCKAR G-128T			CCKAR A-81 G		
	Wild type GG	Mutation GT or TT	p*	Wild type AA	Mutation AG or GG	p
n	1650	601		1317	333	
Age (years)	59.2 ± 0.3†	59.3 ± 0.4	NS‡	59.1 ± 0.3	59.5 ± 0.4	NS
Weight (kg)	57.5 ± 0.2	57.0 ± 0.4	NS	57.6 ± 0.3	57.0 ± 0.3	NS
BMI (kg/m^2)	22.9 ± 0.1	22.9 ± 0.1	NS	22.9 ± 0.1	22.9 ± 0.1	NS
Annual income (%; 54,000 US$ or over)	57.5	58.3	NS	58.3	57.0	NS
Education (%; college or over)	26.9	26.0	NS	27.4	25.6	0.009
Smoking (%; smoker)	22.8	22.8	NS	23.6	21.8	NS
JWAIS-R-SF						
IQ	103.4 ± 0.3	101.6 ± 0.6	0.008	103.6 ± 0.4	102.0 ± 0.5	0.011
Information	9.9 ± 0.1	9.7 ± 0.1	NS	9.9 ± 0.1	9.8 ± 0.1	NS
Picture Completion	10.2 ± 0.1	10.0 ± 0.1	NS	10.2 ± 0.1	10.0 ± 0.1	0.043
Similarities	10.3 ± 0.1	10.1 ± 0.1	NS	10.3 ± 0.1	10.1 ± 0.1	0.051
Digit Symbol	11.7 ± 0.1	11.3 ± 0.1	0.003	11.7 ± 0.1	11.4 ± 0.1	0.008

†Mean ± SE.
‡NS = not significant.
*p-value tested by the t-test or χ^2 test.

genotypes. The IQ levels of subjects with wild-type and mutation alleles at nucleotide -128 were 104.1 ± 0.4 and 102.0 ± 0.6, respectively. There was a significant difference in IQ ($p = 0.002$). The scores of Information and Digit Symbol were significantly lower in subjects with a mutation ($p = 0.012$ and $p = 0.003$, respectively). There were no differences in the scores of Picture Completion and Similarities subtests for polymorphism G-128T. The IQ level was 104.2 ± 0.4 in the subjects with wild-type and 102.6 ± 0.5 in the subjects with mutation at nucleotide -81. Difference in IQ by A-81G polymorphism was significant ($p = 0.008$). Similarities and Digit Symbol subtest scores were significantly lower in subjects with the mutation ($p = 0.033$ and $p = 0.013$, respectively). The Information subtest score was marginally lower with mutation of nucleotide -81 ($p = 0.078$). However, there was no significant difference in the score of Picture Completion subtest.

Haplotype Analysis

Possible haplotypes in the combinations of polymorphism A-81G/G-128T were GA, GG, TG, and TA. However, there were no subjects with AA/GT, AA/TT, or AG/TT genotypic combinations (Table 2). The common haplotype of AA/GT, AA/TT, or AG/TT genotypic combinations was TA. It was considered that no subject had a TA haplotype. The distribution of haplotypes GA, GG, and TG is shown in Table 5. The number of GA haplotypes was 3432; GG was 420; and TG was 650. There was a significant difference in IQ among haplotypes GA, GG, and TG. The IQ for haplotype GA was the highest and the IQ for haplotype TG was the lowest. With an increase in the number of mutation alleles, the IQ level decreased ($p = 0.018$). Digit Symbol scores also significantly decreased with an increasing number of mutation alleles ($p = 0.012$).

TABLE 4. Comparison of intelligences between wild-type and mutation alleles in CCKAR G-81T and A-128G genotypes. Subjects who had used drugs acting on the CNS or subjects with IQ less than 70 were excluded

	CCKAR G-128T			CCKAR A-81G		
	Wild type GG	Mutation GT or TT	p*	Wild type AA	Mutation AG or GG	p
n	1489	539		1178	850	
JWAIS-R-SF						
IQ	104.1 ± 0.4†	102.0 ± 0.6	0.002	104.2 ± 0.4	102.6 ± 0.5	0.008
Information	10.0 ± 0.1	9.6 ± 0.1	0.012	10.0 ± 0.1	9.8 ± 0.1	0.078
Picture Completion	10.2 ± 0.1	10.1 ± 0.1	NS‡	10.3 ± 0.1	10.1 ± 0.1	NS
Similarities	10.4 ± 0.1	10.2 ± 0.1	NS	10.4 ± 0.1	10.2 ± 0.1	0.033
Digit Symbol	11.8 ± 0.1	11.4 ± 0.1	0.003	11.8 ± 0.1	11.5 ± 0.1	0.013

†Mean ± SE.
‡NS = not significant.
*p-value tested by the t-test.

AEP Vol. 15, No. 3
March 2005: 196–201

TABLE 5. Comparison of intelligences between wild-type and mutation alleles in CCKAR G-81T and A-128G genotypes

	Haplotype			
	GA	GG	TG	p for trend*
n	3432	420	650	
JWAIS-R-SF				
IQ	103.2 ± 0.2†	103.0 ± 0.7	101.7 ± 0.6	0.018
Information	10.0 ± 0.1	9.8 ± 0.1	9.7 ± 0.1	NS‡
Picture Completion	10.2 ± 0.1	10.1 ± 0.1	10.0 ± 0.1	NS
Similarities	10.3 ± 0.1	10.1 ± 0.1	10.1 ± 0.1	NS
Digit symbol	11.6 ± 0.1	11.6 ± 0.1	11.3 ± 0.1	0.012

†Mean ± SE.
‡NS = not significant.
*Trend of the three groups was tested by the general linear model.

DISCUSSION

Accumulating data support the involvement of the dopaminergic system in cognitive processing. It is known that CCKAR modulates CCK-stimulated dopamine release in the brain, and mutations in the CCKAR gene may influence the dopaminergic system (5). Considerable preclinical and clinical evidence indicate that inhibitory effects on dopaminergic systems by antipsychotic medications may account for cognitive impairment. A report showed sustained activation of the human mesolimbic dopaminergic system during the performance of cognitive tasks (13). It was also reported that systemic administration of the CCKAR selective antagonist, devazepide, impaired the development of conditioned incentive learning in rats (14). From these data, it is suspected that mutation in the CCKAR gene may influence intelligence.

The CCKAR promoter genotypes were significantly related to IQ. The IQ levels of subjects with the mutant allele were significantly lower than those of subjects with the wild-type allele both for G-128T and A-81G genotypes. A difference in IQ by CCKAR promoter gene polymorphisms was seen in both middle-aged and elderly people. In analyses excluding the subjects who had used drugs acting on the CNS and subjects with IQ less than 70, there was also a significant difference in IQ between the wild and mutation genotypes. We carried out association studies of quantitative traits with haplotypes, and found that the IQ became lower with an increase in the number of mutation alleles.

The CCKAR gene polymorphisms of G-128T and A-81G are located in the promoter region of the gene. It is suspected that mutation of these genotypes is related to the amount of CCKAR production. However, it is still unclear whether these CCKAR polymorphisms are functional or if they are in linkage disequilibrium with other as yet unknown polymorphisms in the CCKAR gene or in a neighboring gene.

In the studies on intelligence in the general population, investigation of genetic factors is an important issue (15). However, at the present time, gene polymorphism has infrequently been reported to be associated with cognition (16). It is suspected that there are many genes associated with individual differences in intelligence, and intelligence is determined from interactions of these gene polymorphisms. However, the contribution of each gene to intelligence may be small as indicated by the results of this study. Testing of thousands of subjects is required to detect small but significant differences. A detailed assessment of IQ requires interviews by psychologists. Assessment of IQ in a large-scale community-dwelling population is generally difficult. It is also difficult to obtain DNA specimens from community-dwelling populations. Because of this, studies on the association between genotype and intelligence have not progressed. In the present study, we showed the relationship between intelligence and CCKAR promoter mutations G-128T and A-81G in community-living middle-aged and elderly Japanese. CCKAR-promoter genotyping may provide useful information for assessing intelligence and preventing cognitive impairment.

REFERENCES

1. Hansen TV. Cholecystokinin gene transcription: Promoter elements, transcription factors, and signaling pathways. Peptides. 2001;22:1201–1211.
2. Hill DR, Shaw TM, Graham W, Woodruff GN. Autoradiographical detection of cholecystokinin-A receptors in primate brain using 125I-Bolton Hunter CCK-8 and 3H-MK-329. J Neurosci. 1990;10:1070–1081.
3. Brenner LA, Ritter RC. Type A CCK receptors mediate satiety effects of intestinal nutrients. Pharmacol Biochem Behav. 1996;54:625–631.
4. Lydiard RB. Neuropeptides and anxiety: Focus on cholecystokinin. Clin Chem. 1994;40:315–318.
5. Tachikawa H, Harada S, Kawanishi Y, Okubo T, Shiraishi H. Novel polymorphisms of the human cholecystokinin A receptor gene: An association analysis with schizophrenia. Am J Med Genet. 2000;96:141–145.
6. Nomoto S, Miyake M, Ohta M, Funakoshi A, Miyasaka K. Impaired learning and memory in OLETF rats without cholecystokinin (CCK)-A receptor. Physiol Behav. 1999;66:869–872.
7. Li XL, Aou S, Hori T, Oomura Y. Spatial memory deficit and emotional abnormality in OLETF rats. Physiol Behav. 2002;75:15–23.
8. Shimokata H, Ando F, Niino N. A new comprehensive study on aging—The National Institute for Longevity Sciences, Longitudinal Study of Aging (NILS-LSA). J Epidemiol. 2000;10:S1–S9.
9. Wechsler D. Wechsler Adult Intelligence Scale – Revised manual. New York: Psychological Corporation; 1981.
10. Shinagawa F, Kobayashi S, Fujita K, Maekawa HH. Japanese Wechsler Adult Intelligence Scale–Revised [in Japanese]. Tokyo: Nihon-Bunka-Kagaku-Sha (Japan Culture and Science Company); 1990.
11. Kobayashi S, Fujita K, Maekawa H, Dairoku H. Short form method in Japan [in Japanese]. In: Misawa G, ed. Japanese Wechsler Adult Intelligence Scale-Revised Short Forms. Tokyo: Nihon-Bunka-Kagaku-Sha (Japan Culture and Science Company); 1993:23–55.
12. Funakoshi A, Miyasaka K, Matsumoto H, Yamamori S, Takiguchi S, Kataoka K, et al. Gene structure of human cholecystokinin (CCK) type-A receptor: Body fat content is related to CCK type-A receptor gene promoter polymorphism. FEBS Lett. 2000;466:264–266.

13. Fried I, Wilson CL, Morrow JW, Cameron KA, Behnke ED, Ackerson LC, et al. Increased dopamine release in the human amygdala during performance of cognitive tasks. Nat Neurosci. 2001;4:201–206.
14. Josselyn SA, Franco VP, Vaccarino FJ. Devazepide, a CCKA receptor antagonist, impairs the acquisition of conditioned reward and conditioned activity. Psychopharmacology. 1996;123:131–143.
15. Bouchard TJ Jr, McGue M. Familial studies of intelligence: A review. Science. 1981;212:1055–1059.
16. Payton A, Holland F, Diggle P, Rabbitt P, Horan M, Davidson Y, et al. Cathepsin D exon 2 polymorphism associated with general intelligence in a healthy older population. Mol Psychiatry. 2003;8:14–18.

J Mol Med (2005) 83:50–57
DOI 10.1007/s00109-004-0578-4

ORIGINAL ARTICLE

Yoshiji Yamada · Fujiko Ando · Naoakira Niino · Hiroshi Shimokata

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

Received: 12 March 2004 / Accepted: 18 June 2004 / Published online: 4 November 2004

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 $(CAG)_{n\leq 22}$ and $(CAG)_{n\geq 23}$ 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.

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

Yoshiji Yamada received his M.D. degree in 1982 and his Ph.D. degree in 1990 in internal medicine from Nagoya University Graduate School of Medicine in Nagoya, Japan. He carried out postdoctoral research at the Eccles Institute of Human Genetics, University of Utah, Salt Lake City, Utah, USA. He is presently Professor and Director of the Department of Human Functional Genomics, Life Science Research Center, Mie University, Tsu, Mie, Japan. His research interests include genomic epidemiology and functional genomics of cardiovascular disease, stroke, osteoporosis, diabetes mellitus, and cancer.

Hiroshi Shimokata received his M.D. degree in 1977 and his Ph.D. degree in 1983 in internal medicine from Nagoya University Graduate School of Medicine in Nagoya, Japan. He carried out postdoctoral research at the National Institute of Aging, NIH, Baltimore, Maryland, USA. He is presently Director of the Department of Epidemiology, National Institute for Longevity Sciences, Obu, Aichi, Japan. His research interests include molecular epidemiology and geriatrics.

Y. Yamada (✉)
Department of Human Functional Genomics,
Life Science Research Center,
Mie University, 1515 Kamihama, Tsu, 514-8507 Mie, Japan
e-mail: yamada@gene.mie-u.ac.jp
Tel.: +81-59-2315387, Fax: +81-59-2315388

F. Ando · N. Niino · H. Shimokata
Department of Epidemiology,
National Institute for Longevity Sciences,
Obu, Aichi, Japan

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 1 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′-GGCGCCGACCAACTTXCC-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 $(CAG)_{n\leq 22}$ and $(CAG)_{n\geq 23}$ 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,

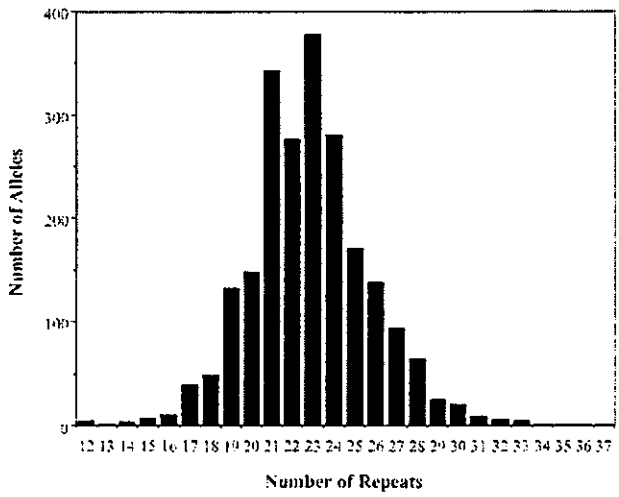

Fig. 1 Distribution of the number of CAG repeats in *AR* in 1,101 women (2,202 alleles)

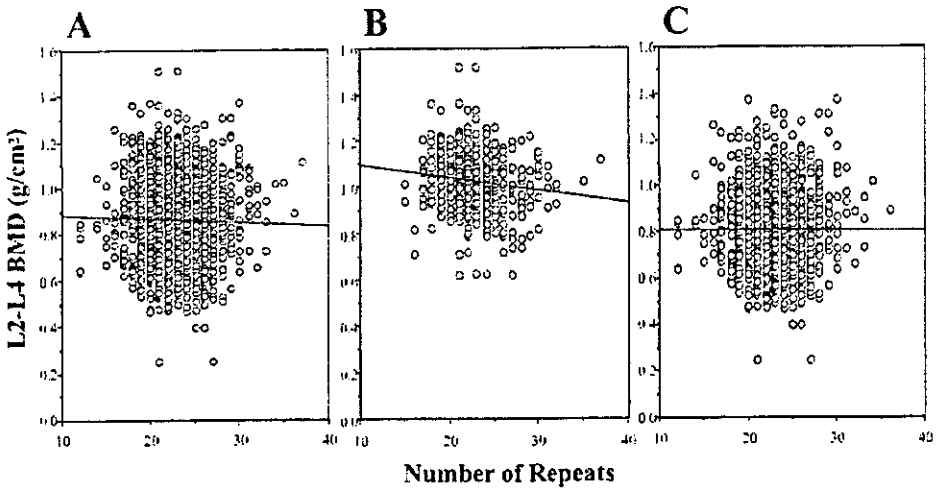

Fig. 2 Relationship between the number of CAG repeats in *AR* and L2-L4 BMD. A All women (n=1,101, 2,202 alleles); r=−0.01967, P=0.3584. B Premenopausal women (n=275, 550 alleles); r=−0.14455, P=0.0007. C Postmenopausal women (n=809, 1,618 alleles); r=0.00751, P=0.7644

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/cm²)					
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

* $P \leq 0.05$, ** $P \leq 0.01$ 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 ($P \leq 0.005$) 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 ($P \leq 0.005$) 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 ($P \leq 0.05$) 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

*$P\leq0.01$, **$P\leq0.005$ vs. *SS*, ***$P\leq0.05$, [4]*§$P\leq0.01$, [5]*$P\leq0.001$ 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

* $P\leq0.05$, **$P\leq0.005$ vs. *GG*, ***$P\leq0.05$ 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

* $P\leq0.05$, **$P\leq0.01$ 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 $(CAG)_{n\geq 23}$ alleles than in those with one or two $(CAG)_{n\leq 22}$ 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 [$(CAG)_{n\leq 19}$] [37] and our study [$(CAG)_{n\leq 22}$] 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 $(CAG)_{n\leq 22}$ and $(CAG)_{n\geq 23}$ 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: $(CAG)_{n\leq 21}$ [24], $(CAG)_{n\leq 20}$ [22], $(CAG)_{n\leq 19}$ [37], and $(CAG)_{n\leq 18}$ [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.

Acknowledgements This work was supported in part by Research Grants for Health and Labor Sciences Research Grants for Comprehensive Research on Aging and Health (H15-Chojyu-014) from the Ministry of Health, Labor, and Welfare of Japan.

References

1. Kanis JA, Melton LJ III, Christiansen C, Johnston CC, Khaltaev N (1994) The diagnosis of osteoporosis. J Bone Miner Res 9:1137–1141
2. Pocock NA, Eisman JA, Hopper JL, Yeates MG, Sambrook PN, Eberl S (1987) Genetic determinations of bone mass in adults: a twin study. J Clin Invest 80:706–710
3. Gueguen R, Jouanny P, Guillemin F, Kuntz C, Pourel J, Siest G (1995) Segregation analysis and variance components analysis of bone mineral density in healthy families. J Bone Miner Res 10:2017–2022
4. Howard GM, Nguyen TV, Harris M, Kelly PJ, Eisman JA (1998) Genetic and environmental contributions to the association between quantitative ultrasound and bone mineral density measurements: a twin study. J Bone Miner Res 13:1318–1327
5. Johnson ML, Gong G, Kimberling W, Recker SM, Kimmel DB, Recker RB (1997) Linkage of a gene causing high bone mass to human chromosome 11 (11q12–13). Am J Hum Genet 60:1326–1332
6. Devoto M, Shimoya K, Caminis J, Ott J, Tenenhouse A, Whyte MP, Sereda L, Hall S, Considine E, Williams CJ, Tromp G, Kuivaniemi H, Ala-Kokko L, Prockop DJ, Spotila LD (1998) First-stage autosomal genome screen in extended pedigrees suggests genes predisposing to low bone mineral density on chromosomes 1p, 2p and 4p. Eur J Hum Genet 6:151–157
7. Econs MJ, Koller DL, Hui SL, Fishburn T, Conneally PM, Johnston CC Jr, Peacock M, Foroud TM (2004) Confirmation of linkage to chromosome 1q for peak vertebral bone mineral density in premenopausal white women. Am J Hum Genet 74:223–228
8. Morrison NA, Qi JC, Tokita A, Kelly PJ, Crofts L, Nguyen TV, Sambrook PN, Eisman JA (1994) Prediction of bone density from vitamin D receptor alleles. Nature 367:284–287
9. Uitterlinden AG, Burger H, Huang Q, Yue F, McGuigan FEA, Grant SFA, Hofman A, van Leeuwen JPTM, Pols HAP, Ralston SH (1998) Relation of alleles of the collagen type Iα1 gene to bone density and the risk of osteoporotic fractures in postmenopausal women. N Engl J Med 338:1016–1021

10. Yamada Y, Ando F, Niino N, Shimokata H (2001) Transforming growth factor-β1 gene polymorphism and bone mineral density. JAMA 285:167–168
11. Sowers M, Willing M, Burns T, Deschenes S, Hollis B, Crutchfield M, Jannausch M (1999) Genetic markers, bone mineral density, and serum osteocalcin levels. J Bone Miner Res 14:1411–1419
12. Kawano K, Ogata N, Chisano M, Molloy H, Kleyn P, Spector TD, Uchida M, Hosoi T, Suzuki T, Orimo H, Inoue S, Nabeshima Y, Nakamura K, Kuro-o M, Kawaguchi H (2002) Klotho gene polymorphisms associated with bone density of aged postmenopausal women. J Bone Miner Res 17:1744–1751
13. Vanderschueren D, Bouillon R (1995) Androgens and bone. Calcif Tissue Int 56:341–346
14. Pederson L, Kremer M, Judd J, Pascoe D, Spelsberg TC, Riggs BL, Oursler MJ (1999) Androgens regulate bone resorption activity of isolated osteoclasts in vitro. Proc Natl Acad Sci USA 96:505–510
15. Platz EA, Rimm EB, Willett WC, Kantoff PW, Giovannucci E (2000) Racial variation in prostate cancer incidence and in hormonal system markers among male health professionals. J Natl Cancer Inst 92:2009–2017
16. Hsing AW, Gao Y-T, Wu G, Wang X, Deng J, Chen Y-L, Sesterhenn IA, Mostofi FK, Benichou J, Chang C (2000) Polymorphic CAG and GGN repeat lengths in the androgen receptor gene and prostate cancer risk: a population-based case-control study in China. Cancer Res 60:5111–5116
17. Chamberlain NL, Driver ED, Miesfeld RL (1994) The length and location of CAG trinucleotide repeats in the androgen receptor N-terminal domain affect transactivation function. Nucleic Acids Res 22:3181–3186
18. Kazemi-Esfarjani P, Trifiro MA, Pinsky L (1995) Evidence for a repressive function of the long polyglutamine tract in the human androgen receptor: possible pathogenetic relevance for the (CAG) n-expanded neuronopathies. Hum Mol Genet 4:523–527
19. Tut TG, Ghadessy FJ, Trifiro MA, Pinsky L, Yong EL (1997) Long polyglutamine tracts in the androgen receptor are associated with reduced trans-activation, impaired sperm production, and male infertility. J Clin Endocrinol Metab 82:3777–3782
20. Mhatre AN, Trifiro MA, Kaufman M, Kazemi-Esfarjani P, Figlewicz D, Rouleau G, Pinsky L (1993) Reduced transcriptional regulatory competence of the androgen receptor in X-linked spinal and bulbar muscular atrophy. Nat Genet 5:184–188
21. Zitzmann M, Brune M, Kornmann B, Gromoll J, Junker R, Nieschlag E (2001) The CAG repeat polymorphism in the androgen receptor gene affects bone density and bone metabolism in healthy males. Clin Endocrinol (Oxf) 55:649–657
22. Langdahl BL, Stenkjaer L, Carstens M, Tofteng CL, Eriksen EF (2003) A CAG repeat polymorphism in the androgen receptor gene is associated with reduced bone mass and increased risk of osteoporotic fractures. Calcif Tissue Int 73:237–243
23. Chen H-Y, Chen W-C, Wu M-C, Tsai F-J, Tsai C-H (2003) Androgen receptor (AR) gene microsatellite polymorphism in postmenopausal women: correlation to bone mineral density and susceptibility to osteoporosis. Eur J Obstet Gynaecol Reprod Biol 107:52–56
24. Tofteng CL, Kindmark A, Brändström H, Abrahamsen B, Petersen S, Stiger F, Stilgren LS, Jensen JEB, Vestergaard P, Langdahl BL, Mosekilde L (2004) Polymorphisms in the CYP19 and AR genes—relation to bone mass and longitudinal bone changes in postmenopausal women with or without hormone replacement therapy: the Danish Osteoporosis Prevention Study. Calcif Tissue Int 74:25–34
25. Salmén T, Heikkinen A-M, Mahonen A, Kröger H, Komulainen M, Pallonen H, Saarikoski S, Honkanen R, Mäenp PH (2003) Relation of androgen receptor gene polymorphism to bone mineral density and fracture risk in early postmenopausal women during a 5-year randomized hormone replacement therapy trial. J Bone Miner Res 18:319–324
26. Remes T, Väisänen SB, Mahonen A, Huuskonen J, Kröger H, Jurvelin JS, Penttilä IM, Rauramaa R (2003) Aerobic exercise and bone mineral density in middle-aged Finnish men: a controlled randomized trial with reference to androgen receptor, aromatase, and estrogen receptor α gene polymorphisms. Bone 32:412–420
27. Irvine RA, Yu MC, Ross RK, Coetzee GA (1995) The CAG and GGC microsatellites of the androgen receptor gene are in linkage disequilibrium in men with prostate cancer. Cancer Res 55:1937–1940
28. Kuro-o M, Matsumura Y, Aizawa H, Kawaguchi H, Suga T, Utsugi T, Ohyama Y, Kurabayashi M, Kaname T, Kume E, Iwasaki H, Iida A, Shiraki-Iida T, Nishikawa S, Nagai R, Nabeshima Y (1997) Mutation of the mouse klotho gene leads to a syndrome resembling ageing. Nature 390:45–51
29. Kawaguchi H, Manabe N, Miyaura C, Chikuda H, Nakamura K, Kuro-o M (1999) Independent impairment of osteoblast and osteoclast differentiation in klotho mouse exhibiting low-turnover osteopenia. J Clin Invest 104:229–237
30. Matsumura Y, Aizawa H, Shiraki-Iida T, Nagai R, Kuro-o M, Nabeshima Y (1998) Identification of the human klotho gene and its two transcripts encoding membrane and secreted klotho protein. Biochem Biophys Res Commun 242:626–630
31. Ogata N, Matsumura Y, Shiraki M, Kawano K, Koshizuka Y, Hosoi T, Nakamura K, Kuto-o M, Kawaguchi H (2002) Association of klotho gene polymorphism with bone density and spondylosis of the lumbar spine in postmenopausal women. Bone 31:37–42
32. Shimokata H, Ando F, Niino N (2000) A new comprehensive study on aging—the National Institute for Longevity Sciences, Longitudinal Study of Aging (NILS-LSA). J Epidemiol 10:S1–S9
33. Yamada Y, Ando F, Niino N, Shimokata H (2003) Association of polymorphisms of interleukin-6, osteocalcin, and vitamin D receptor genes, alone or in combination, with bone mineral density in community-dwelling Japanese women and men. J Clin Endocrinol Metab 88:3372–3378
34. Yamada Y, Izawa H, Ichihara S, Takatsu F, Ishihara H, Hirayama H, Sone T, Tanaka M, Yokota M (2002) Prediction of the risk of myocardial infarction from polymorphisms in candidate genes. N Engl J Med 347:1916–1923
35. Irvine RA, Ma H, Yu MC, Ross RK, Stallcup MR, Coetzee GA (2000) Inhibition of p160-mediated coactivation with increasing androgen receptor polyglutamine length. Hum Mol Genet 9:267–274
36. Hsiao P-W, Lin D-L, Nakao R, Chang C (1999) The linkage of Kennedy's neuron disease to ARA24, the first identified androgen receptor polyglutamine region-associated coactivator. J Biol Chem 274:20229–20234
37. Westberg L, Baghaei F, Rosmond R, Hellstrand M, Landén M, Jansson M, Holm G, Björntorp P, Eriksson E (2001) Polymorphisms of the androgen receptor gene and the estrogen receptor β gene are associated with androgen levels in women. J Clin Endocrinol Metab 86:2562–2568
38. Buller RE, Sood AK, Lallas T, Buekers T, Skilling JS (1999) Association between nonrandom X-chromosome inactivation and BRCA1 mutation in germline DNA of patients with ovarian cancer. J Natl Cancer Inst 91:339–346
39. Vottero A, Stratakis CA, Ghizzoni L, Longui CA, Karl M, Chrousos GP (1999) Androgen receptor-mediated hypersensitivity to androgens in women with nonhyperandrogenic hirsutism: skewing of X-chromosome inactivation. J Clin Endocrinol Metab 84:1091–1095
40. Kristiansen M, Langerød A, Knudsen GP, Weber BL, Børresen-Dale A-L, Ørstavik KH (2002) High frequency of skewed X inactivation in young breast cancer patients. J Med Genet 39:30–33

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

Kyoko Miyasaka, MD, PhD, Takako Kawanami, PhD,† Hiroshi Shimokata, MD, PhD,‡ Shigeo Ohta, MD, PhD,§ and Akihiro Funakoshi, MD, PhD†*

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

(*Pancreas* 2005;30:95–98)

Received for publication May 17, 2004; accepted August 30, 2004.

From the *Department of Clinical Physiology, Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan; †Department of Gastroenterology, National Kyushu Cancer Center, Fukuoka, Japan; ‡Department of Epidemiology, National Institute for Longevity Sciences, Ohbu, Japan; and §Department of Biochemistry and Cell Biology, Institute of Gerontology, Nippon Medical School, Kanagawa, Japan.

Reprints: Kyoko Miyasaka, Department of Clinical Physiology, Tokyo Metropolitan Institute of Gerontology, 35-2 Sakaecho Itabashiku, Tokyo 173-0015, Japan (e-mail: miyasaka@tmig.or.jp).

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.

The 70 male subjects (mean age 62 years; range, 41–80) and 44 female subjects (mean age, 66 years; range, 43–93) had been consecutively hospitalized at the National Kyushu Cancer Center. Pancreatic cancer was diagnosed clinically by imaging techniques including ultrasound, CT scanning, and magnetic resonance tomography and was proved by histologic examination.

The age-matched control subjects consisted of 1050 male participants (mean age, 59 years; range, 40–79) and 1020 female participants (mean age, 58 years; range, 40–79) in the NILS Longitudinal Study of Aging (LSA).[14]

Subjects who consumed more than 5 g of ethanol per day were judged as having a drinking habit. The smoking status classifications were current smoker, ex-smoker, and never smoked. Only current smokers were judged as having a smoking habit.

Genotyping Procedures

The genotype of the ALDH2 gene was determined by a mismatched PCR-restriction fragment length polymorphism (RFLP) method reported previously.[15]

Statistical Analysis

Statistical differences between pancreatic cancer subjects and control subjects were assessed using the χ^2 test or Fisher direct test. Probability differences of $P < 0.05$ were considered statistically significant.

RESULTS

Smoking and/or Drinking Habits

The frequency of male subjects who had both smoking and drinking habits was significantly higher in the pancreatic cancer patients than in control subjects (Table 1). The frequency of male subjects who had a smoking habit with or without a drinking habit was significantly higher in pancreatic cancer patients than in control subjects (70% for pancreatic cancer patients vs. 37.5% for controls), whereas a drinking habit with or without a smoking habit was not different between the 2 groups (64.5% for pancreatic cancer patients vs. 66.7% for controls) (Table 1).

In contrast, more than 70% of female subjects had neither habit (Table 1). Although the frequency of subjects who had a smoking habit alone tended to be higher and the frequency of subjects who had a drinking habit alone tended to be lower in pancreatic cancer patients than control subjects regardless of sex, but the differences were not statistically significant.

Distribution of ALDH2 Genotype Between Pancreatic Cancer Patients and Control Subjects

The distribution of the ALDH2 genotype in the control subjects of both sexes was similar to those in previous reports[10] (Table 2). In male subjects, the frequency of the active form of ALDH2 was significantly lower in pancreatic cancer patients than in control subjects ($P < 0.02$), whereas no difference was observed in female subjects in pancreatic cancer patients and control subjects (Table 2).

Smoking and Drinking Habits, and ALDH2 Gene Polymorphism Between Pancreatic Cancer Patients and Control Subjects

In male subjects, the frequency of subjects who had both smoking and drinking habits was significantly higher among the pancreatic cancer patients than among control subjects with either ALDH2*1/2*1 or ALDH2*1/2*2 (Table 3). The odds ratio was 3.13 for the subjects with ALDH2*1/2*1 and 3.12 for those with ALDH2*1/2*2. The frequency of subjects with ALDH2*1/2*2 who had a smoking habit alone was significantly higher in pancreatic cancer patients than in control subjects ($P = 0.048$) (Table 2). On the other hand, none of the 8 pancreatic cancer patients with ALDH2*2/2*2 had a drinking habit. Four of the ALDH2*2/2*2 subjects had a smoking habit (Table 3), but the difference between the pancreatic cancer patients and the controls was not significant ($P = 0.44$) because the absolute number of subjects with ALDH2*/2* was small. However, among the subjects with inactive ALDH2 (including ALDH2*1/2*2 and ALDH2*2/2*2), the frequency of the smoking habit alone was again significantly higher in pancreatic cancer patients than in control subjects ($P < 0.03$).

In contrast, in female subjects because few subjects had smoking and/or drinking habits, there were no significant differences between pancreatic cancer patients and control subjects in terms of habits and/or ALDH2 genotypes (Table 4).

TABLE 1. Smoking and/or Drinking Habits in Pancreatic Cancer Patients and Control Subjects

	Both Smoking and Drinking Habits n (%)	Smoking Habit Alone n (%)	Drinking Habit Alone n (%)	Neither Habit n (%)	Total n (%)
Male					
Pancreatic cancer	35 (50.0)*	14 (20.0)	10 (14.3)	11 (15.7)	70 (100)
Control subjects	294 (27.5)	107 (10.0)	419 (39.2)	233 (21.8)	1050 (100)
Female					
Pancreatic cancer	1 (2.2)	5 (11.4)	3 (6.8)	35 (79.5)	44 (100)
Control subjects	25 (2.5)	46 (4.5)	227 (22.3)	722 (70.8)	1020 (100)

*The frequency was significantly lower compared with that in control subjects ($\chi^2 = 23.01$, df = 1, $P = 0.000$).
The difference between pancreatic cancer patients and control subjects was tested by 2 × 2 χ^2 test.