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Association of bone mineral density with a dinucleotide repeat polymorphism at the calcitonin (*CT*) locus

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Abstract Calcitonin (CT), a calcium-regulating hormone, lowers the calcium level in serum by inhibiting bone resorption. Because CT may play a role in the pathogenesis of osteoporosis, genetic variations in or adjacent to the *CT* gene may be associated with variations in bone mineral density (BMD). The present study examined the correlation between a dinucleotide (cytosine-adenine; CA) repeat polymorphism at the *CT* locus and BMD in 311 Japanese postmenopausal women (mean age, 64.1 years). Seven alleles were present in this population; each allele contained 10, 11, 16, 17, 18, 19, or 20 CA repeats. Thus, we designated the respective genotypes A10, A11, A16, A17, A18, A19, and A20. The A10 and A17 alleles were the predominant alleles in the population studied. Z scores (a parameter representing deviation from the age-specific weight-adjusted average BMD) were compared between individuals that possessed one or two alleles of each genotype and those that did not possess the allele. Subjects who possessed one or two A10 alleles had lower BMD Z scores than those who did not (lumbar 2–4 BMD Z score; -0.148 ± 1.23 vs 0.182 ± 1.54; $P = 0.04$). No significant relationships were observed between allelic status and background data or biochemical parameters. The significant association observed between BMD and genetic variations at the *CT* locus implies that polymorphism at this locus may be a useful marker for the genetic study of osteoporosis.

Key words Calcitonin gene · Bone mineral density · Osteoporosis · Microsatellite polymorphism · Risk factors

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Introduction

Osteoporosis is characterized by low bone mass and by the microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture (Consensus development conference 1993). Bone mineral density (BMD) is the primary factor affecting susceptibility to fracture and is determined by many genetic and lifestyle factors. Its predictive value is strongly supported by twin (Dequeker et al. 1987; Kelly et al. 1993; Pocock et al. 1987; Slemenda et al. 1991) and familial studies (Krall and Dawson-Hughes 1993; Lutz 1986; Seeman et al. 1989; Tylavsky et al. 1989). Knowing the genetic risk factors for an individual would assist in the diagnosis, prevention, and therapy of osteoporosis. Some genetic effects have been ascribed to polymorphisms of genes involved in bone metabolism, including the vitamin D receptor (*VDR*) gene (Morrison et al. 1994), the estrogen receptor (*ER*) gene (Kobayashi et al. 1996; Sano et al. 1995), the type I collagen gene (Grant et al. 1996), the apolipoprotein E gene (Shiraki et al. 1997), the transforming growth factor β gene (Langdahl et al. 1997), the parathyroid hormone (*PTH*) gene (Hosoi et al. 1998), and the interleukin 6 gene (Emi et al. 1999). However, whether these polymorphisms actually affect BMD is a matter of controversy.

Many endocrinological factors are known to play roles in bone maturation and in the process of bone loss that accompanies aging (Raisz 1988). Because it is entirely possible that the pathophysiology or key genetic background of each osteoporotic patient is heterogeneous, a rational approach to an understanding of the genetic background of osteoporosis would require an expansion of the panel of genes examined.

Calcitonin (CT) is a polypeptide containing 32 amino acids that are synthesized by the parafollicular cells of the thyroid (Fischer and Born 1987). CT lowers serum calcium by inhibiting bone resorption and promoting the renal excretion of calcium. Increased bone resorption causes bone loss during aging in postmenopausal women. CT inhibits bone loss in estrogen-deficient individuals and is thought to suppress bone turnover (Gennari et al. 1992; MacIntyre et al. 1988; Owan and Ibaraki 1994).

A young osteoporotic man was reported to have an undetectable plasma CT concentration and a single base insertion in the intron separating exons IV and V of the *CT* gene (Alevizaki et al. 1989). This evidence suggests that genetic variation in the *CT* gene may contribute to the pathogenesis of osteoporosis. Recent studies (Masi et al. 1998; Taboulet et al. 1998) have shown a relationship between polymorphisms of the CT receptor gene and bone mineral density. However, no correlation between genetic variations in the *CT* gene itself and bone metabolism has yet been shown, partly because no useful genetic markers for this gene have been recognized.

Recently, dinucleotide repeat polymorphic markers, or microsatellites, have been used to analyze multifactorial diseases (Houseman 1995). Microsatellite polymorphism can be employed for family-based studies, including sib-pair analysis, and population-based association studies. In the present study, we used a newly isolated dinucleotide (cytosine-adenine; CA) repeat polymorphism at the *CT* gene locus (Tsukamoto and Emi 1998) to analyze the relationship between allelic status and BMD in postmenopausal Japanese women.

Subjects and methods

Subjects

Genotype analysis was performed on samples collected from 311 healthy postmenopausal Japanese women (age, 64.1 ± 8.2 years; mean ± SD), who were recruited from among unrelated volunteers living in Nagano prefecture, Japan. DNA samples were obtained from the peripheral blood of subjects as previously described (Emi et al. 1999). Exclusion criteria for the present study included the presence of endocrinological disorders (such as hyperthyroidism, hyperparathyroidism, and diabetes mellitus); liver disease; or renal disease; the use of medicines known to affect bone metabolism (such as corticosteroids, anticonvulsants, heparin); or an unusual gynecological history. None of the women who participated in the present study were related, and all of the women were volunteers who gave their informed consent.

Measurement of bone mineral density (BMD) and biochemical markers

The spine BMD and total body BMD (BMD in g/cm^2) of each participant were measured using dual-energy X-ray absorptiometry (DPX-L; Lunar, Madison, WI, USA). The following biochemical parameters were measured, as described elsewhere (Shiraki 1997): serum concentration of calcium (Ca), phosphate (P), alkaline phosphatase (ALP), intact osteocalcin (I-OC; enzyme-linked immunosorbent assay [ELISA], Teijin, Tokyo, Japan), N-fragment osteocalcin (N-OC; ELISA, Teijin), intact parathyroid hormone (intact PTH; IRMA kit, Nichols Institute Diagnostics, San Juan Capistrano, CA, USA), and calcitonin (CT; ELISA, Mitsubishi Chemical, Tokyo, Japan). The minimum detectable level of CT was 12.5 pg/ml. In addition, the calcium/creatine ratio, the phosphate/creatinine ratio, the amount of pyridinoline (Pyr; by the HPLC method), and the amount of deoxypyridinoline (Dpyr; by the HPLC method) present in the urine of each woman were measured. Z scores (a parameter representing deviation from the age-specific weight-adjusted average BMD) were used to analyze the BMD data. Data from 20,000 Japanese women were used to calculate Z scores, using Lunar DPX-L installed software.

Determination of microsatellite polymorphism

Isolation and characterization of a microsatellite polymorphism at the *CT* locus was reported by us (Tsukamoto and Emi 1998). This polymorphism was genotyped by polymerase chain reaction (PCR), using Cy5-labeled forward primer (CT-4F:5′-GGAGACAAACAGGGATGACA-3′, Pharmacia Amersham, Tokyo, Japan) and unlabeled reverse primer (CT-4R:5′-CAGAAACATGGTGTGCCA GC-3′). The PCR reaction was carried out in a final volume of 25 μl containing 100 ng of genomic DNA obtained from peripheral white blood cells, 10 pmol of each primer, 200 mM dNTP, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 0.001% gelatin, and 0.1 U Taq DNA polymerase (Ampli Taq Gold; PE Biosystems, Tokyo, Japan) (Tsukamoto et al. 1998; Watanabe et al. 1998). Thirty PCR cycles (each 30 s at 94°C, 60 s at 58°C, and 60 s at 72°C) were performed (Hirayama et al. 1998). The resulting PCR products were then electrophoresed in a 2% agarose gel. An aliquot from each reaction was mixed with formamide/blue dextran gel-loading buffer, internal size marker (100 bp, 200 bp; Pharmacia Amersham), heated at 95°C for 5 min, and cooled on ice for 5 min. Subsequently, the products were separated on a 6% urea gel (6% Long Ranger; Takara Shuzou, 6 M Urea, 0.5 × Tris Bolate EDTA [TBE]) at 55 W for 700 min, using an ALFexpress DNA sequencer (Pharmacia Amersham).

A genomic DNA homozygous for the most frequent size was subcloned in PCRII plasmid (Invitrogen, Carlsbad, CA, USA) and sequenced using an autosequencer equipped with an Autoread sequencing kit (Pharmacia Amersham); this contained 17(CA) repeats and the allele was named A17. The sizes of other PCR products were determined through comparison with the sequenced sample named A17, using Fragment Manager software (Pharmacia Amersham). We determined the names of the other samples comparing the size with A17.

Statistical analysis

Non-parametric Student-Neumann-Klaus *t*-test has been routinely used as a standard method for statistical analysis in correlating allelic status with quantitative traits, such as BMD, blood pressure (Sano et al. 1995; Kobayashi et al. 1996; Shiraki et al. 1997; Hosoi et al. 1998; Tsukamoto et al. 1999; Ogawa et al. 2000). In the present study, we followed this convention of analyzing quantitative traits by Student-Newman-Klaus *t*-test. Therefore, comparisons of Z scores and biochemical markers between the group of individuals possessing one or two alleles of each genotype and the group that did not possess that genotype were performed using Student's *t*-test (StatView-J 4.5, Abacus Concepts, Tokyo, Japan). A *P* value less than 0.05 was considered significant. Other methods, including the χ^2 test, Wilcoxon signed-ranks test, and analysis of variance, may also be used occasionally; for example, the χ^2 test is used to analyze deviations of genotype frequencies from Hardy-Weinberg expectation in case-control comparisons.

Results

Using molecular size markers and the installed software, we determined the number of CA repeats for each allele by DNA sequencing. PCR products were shown to contain 10, 11, 16, 17, 18, 19, or 20 CA repeats. We designated these alleles as A10, A11, A16, A17, A18, A19, and A20, respectively, and determined the genotype of each of the 311 postmenopausal Japanese women. The allele distribution among the 622 chromosomes is shown in Fig. 1. CA repeats 10 and 17 were the dominant types.

The subjects were then grouped according to genotype, and their BMD and BMD Z scores were statistically compared between the group with a given genotype and all subjects without that genotype. The Z score for lumbar BMD was significantly different between subjects with the A10 genotype and those without A10 (Fig. 2). Other genotypes did not appear to affect the BMD Z scores. We then compared demographic and baseline data between the groups classified as A10 and non-A10 and found no significant differences (Table 1). Similarly, comparisons of biochemical parameters, including bone metabolic parameters and serum CT concentration, revealed no significant differences (Table 2). Of the 147 women possessing the A10 allele, 25 were homozygous at this locus. However, no significant differences in the BMD Z score were detected between women who were homozygous for the A10 allele and those who were heterozygous (data not shown).

Table 1. Comparison of background data in Japanese menopausal women with and without an A10 allele at the *CT* locus

	A10 (+) (n = 147)	A10 (−) (n = 161)	*P* value
Age (years)	65.2 ± 9.4	64.1 ± 8.1	0.42
Years after menopause	15.5 ± 9.1	14.5 ± 8.3	0.25
Age at menarche (years)	16.0 ± 1.7	15.4 ± 1.8	0.16
BH (cm)	151.2 ± 5.6	150.7 ± 6.1	0.59
BW (kg)	52.2 ± 8.4	51.2 ± 7.9	0.92
Cr (mg/dl)	0.65 ± 0.29	0.76 ± 0.24	0.54

All data values are expressed as means ± SD. There were no differences between the groups
BH, Body height; BW, body weight; Cr, creatinine; CT, calcitonin

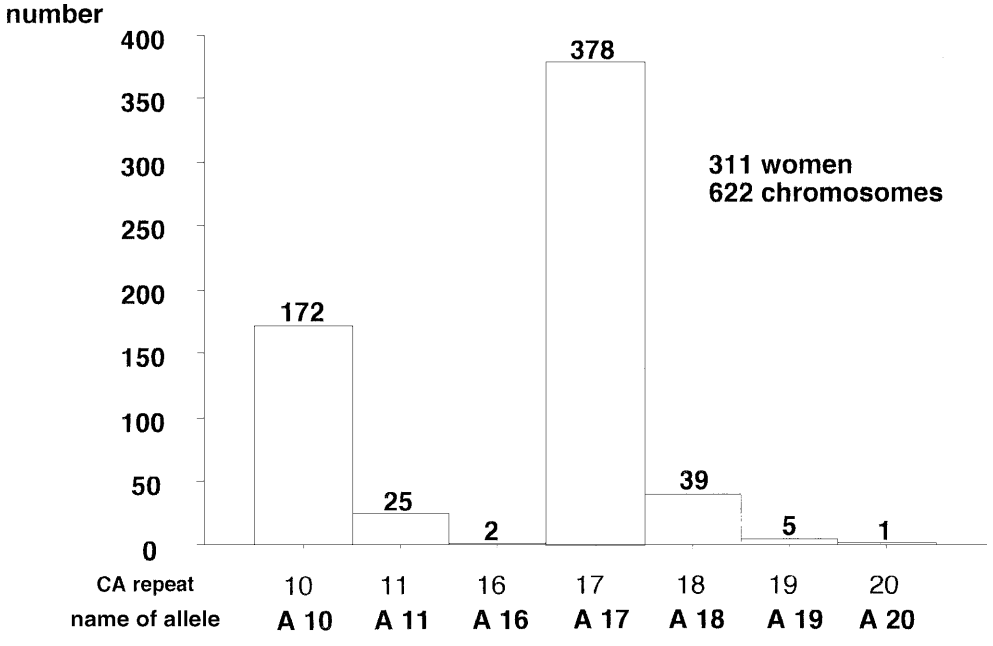

Fig. 1. Frequency distribution of *calcitonin* (*CT*) dinucleotide repeat polymorphism in Japanese postmenopausal women *CA*, Cytosine-adenine

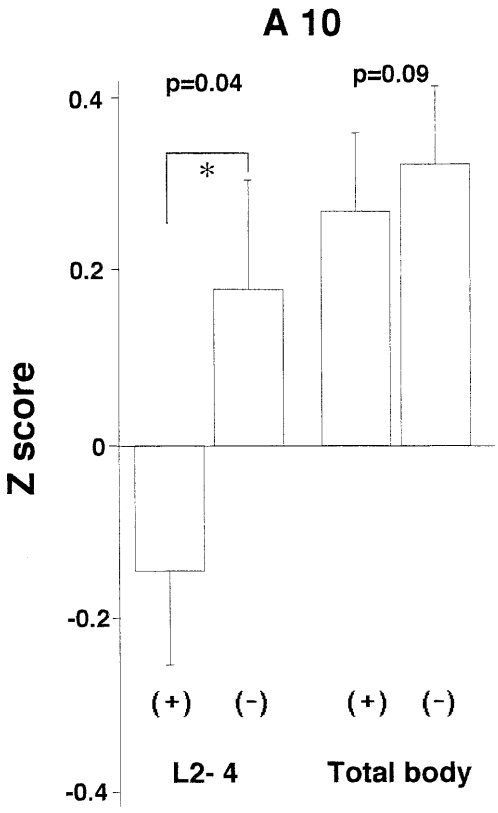

Fig. 2. Comparison of bone mineral density (BMD) between subjects with and without the *CT* A10 allele. Z score values for lumbar spine BMD (*L2–4*) and total body BMD (*total body*) were compared between the subjects with (+) and without (−) a *CT* allele. *P* values are shown when they are less than 0.10. The difference between A10 (+) and A10 (−) was significant. (*$P = 0.04$). See text for explanation of 2 score

Table 2. Effects of carriage of the A10 allele on bone metabolic markers and calcium-regulating hormones

	A10 (+) ($n = 147$)	A10 (−) ($n = 164$)	*P* value
Intact-OC (ng/ml)	7.1 ± 3.4	7.8 ± 3.7	0.12
U-pyr (pmol/μmol)	33.4 ± 10.4	33.5 ± 9.9	0.90
U-deoxypyr. (pmol/μmol)	7.2 ± 2.3	7.2 ± 2.3	0.87
Intact PTH (pg/ml)	37.0 ± 13.9	36.7 ± 13.2	0.35
Calcitonin (pg/ml)	23.0 ± 10.8	24.1 ± 9.6	0.37
1, 25 $(OH)_2D_3$ (ng/ml)	32.4 ± 13.2	33.8 ± 11.6	0.35

All data values are expressed as means ± SD. The *CT* genotype, A10, did not affect bone metabolic markers or calcium-regulating hormone Intact-OC, intact-osteocalcin; U-pyr, urinary pyridinoline; U-deoxypry., urinary deoxypyridinoline; intact PTH, intact parathyroid hormone; 1,25 $(OH)_2D_3$, 1,25-dehydroxyvitamin D3

Discussion

The present study used a newly isolated dinucleotide repeat polymorphism adjacent to the *CT* gene to associate an allelic genotype, A10, with lower BMD in Japanese postmenopausal women. The data presented in this report suggest that variation or mutation in or adjacent to the *CT* gene may affect bone metabolism and eventually cause variations in BMD. However, because our study population consisted of a single ethnic group and was rather small in scale, it would be of value to have further confirmation in different populations to allow definitive conclusions to be drawn regarding the association between CT and BMD.

The *CT* gene is located at 11p15.1–15.2 (Hoovers et al. 1993). Calcitonin gene-related peptide (CGRP) is an alternative splicing product of the *CT* gene transcript (Rosenfeld et al. 1983). Recently, neuronal factors, in particular, neuropeptides, have been reported to regulate bone cells (Konttinen et al. 1996). CGRP has been reported to recruit macrophages into osteoclast-like cells (Owan and Ibaraki 1994), to inhibit bone resorption (Roos et al. 1986), and to stimulate osteogenesis (Bernard and Shih 1990). It is possible that the allelic genotype associated with the lower BMD in our test subjects may be in linkage disequilibrium with a mutation in *CGRP*; another possibility is involvement of the gene encoding PTH, which lies in the same genomic vicinity as *CT* (Kittur et al. 1985). This spatial relationship is intriguing when we consider the importance of both hormones in calcium metabolism.

No significant differences were observed in bone metabolic markers, including serum CT, between the group with A10 and the group without this genotype. In this study, the minimum detectable level of CT (12.5 pg/ml) was relatively high; a total of 58 subjects (18.6%) had CT under this level, and the frequency distribution of subjects with an undetectable level in each group was not different (data not shown). A more sensitive assay of CT would help to elucidate this issue.

It is well established that CT is useful in the treatment of osteoporosis (Gennari et al. 1992; MacIntyre et al. 1988; Owan and Ibaraki 1994). Moreover, a report of *CT* gene mutation in a young patient with osteoporosis also supports the idea that CT is important in maintaining bone mass (Alevizaki et al. 1989). Although the correlation between CT plasma level and BMD remains unclear (Chesnut et al. 1980; Leggate et al. 1984; Prince et al. 1989), we can speculate that a variation in the *CT* locus may be correlated to variations in the effect of CT in maintaining calcium homeostasis. For example, the CT secretory reaction to calcium infusion in osteoporotic women was reported to be lower than that in normal women (Taggart et al. 1982). In addition, it was reported that CT secretion response decreased with aging (Deftos et al. 1980). Clarification of the relationship between the *CT* gene and osteoporosis requires further knowledge of the regulatory mechanisms of gene expression and post-translational processing of *CT* gene. To that end, a future search for single nucleotide polymorphisms (SNPs) that may affect calcium metabolism, as well as functional studies of the involvement of these SNPs in the pathogenesis of osteoporosis, would be warranted.

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