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ASSOCIATION OF ESTROGEN RECEPTOR DINUCLEOTIDE REPEAT POLYMORPHISM WITH OSTEOPOROSIS

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SUMMARY: We investigated the association between dinucleotide (thymine-adenine) repeat polymorphism lying upstream of human estrogen receptor (ER) gene and bone mineral density (BMD) as well as biochemical markers for bone metabolism in 144 healthy postmenopausal Japanese women. The genotype was classified into 'A' through 'R' according to the number of the repeats, from 10 to 27. BMD was expressed in Z score (a deviation from the weight-adjusted average BMD of each age using the standard deviation as a unit). The people having genotype C (12 repeats of thymine-adenine) allele (n=15) had significantly lower Z score of spine BMD (mean ± SD; -1.11 ± 1.3 vs. -0.06 ± 1.2; $p<0.01$) and of total body BMD (-0.58 ± 1.0 vs. 0.31 ± 0.9; $p<0.01$) than those without this genotype (n=129). They also had significantly higher levels of serum intact osteocalcin, urinary pyridinoline, and urinary deoxypyridinoline. These results suggest that genetic variation at the ER locus may be associated with some determinants for BMD and bone metabolism in postmenopausal women.

It is well established that estrogen deficiency plays a major role in the pathogenesis of postmenopausal osteoporosis (1) which is characterized by the pathologically low bone mass and an increased risk for fracture. This is confirmed by the fact that estrogen replacement therapy has

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Abbreviations: ER, estrogen receptor; BMD, bone mineral density; TA, thymine-adenine; PCR, polymerase chain reaction; I-OC, intact osteocalcin; Pyr, pyridinoline; Dpyr, deoxypyridinoline.

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significant effects in the prevention and treatment of osteoporosis (1-3). It has been revealed that not only osteoblasts but also osteoclasts express estrogen receptor (ER) (4-7). These evidences support the hypothesis that estrogen affects directly bone metabolism.

Rates of postmenopausal bone loss are heterogeneous among individuals, ranging from "normal" to "rapid" bone loss. On the other hand, heritability of bone mass has been suggested in the studies of twin (8,9). It is possible that the heterogeneity in bone mass and bone metabolism may reflect genetic variation determining the responsiveness to lower level of estrogen in postmenopausal period. However, little is known to explain this hypothesis.

In the study, we investigated the relationship between bone metabolism and genetic markers at the ER gene locus in Japanese postmenopausal women, using a "microsatellite" containing a thymine-adenine (TA) repeat polymorphism that is located in the genomic region upstream of the human ER gene (10).

MATERIALS AND METHODS

Subjects

Genotype analysis was done using the samples obtained from 274 healthy postmenopausal Japanese women living in the Nagano area. Whole sets of clinical data described below were available about 144 subjects (ages 45-91 years, mean age 65.6 ± 10.5 years) out of the 274 women. All were non-related volunteers and gave their informed consent prior to the study. None had medical complications or unusual gynecological history and were under medical treatment that were known to affect bone metabolism (such as hyperthyroidism, renal disease, or collagen disease).

Measurement of bone mineral density (BMD)and biochemical markers

Spine BMD and total body BMD (BMD in g/cm^2) of each participant were measured by dual-energy X-ray absorptiometry (DPX-L, Lunar Co., USA). The following biochemical parameters were measured; serum concentration of calcium (Ca), phosphate (P), alkaline phosphatase, intact osteocalcin (I-OC, ELISA, Teijin Co., Ltd., Tokyo, Japan), N-fragment osteocalcin, parathyroid hormone and calcitonin. Calcium/creatine ratio, Phosphate/creatinine ratio, pyridinoline (Pyr, HPLC method) and deoxypyridinoline (Dpyr, HPLC method) in urine were also measured. Z score was utilized in order to analyze the data of BMD, which is a deviation from the weight-adjusted average BMD of each age. Z scores were calculated using the installed software of Lunar DPX-L based on 20,000 Japanese women's data.

Determination of microsatellite polymorphism by polymerase chain reaction

The polymerase chain reaction (PCR) was performed using oligonucleotide primers designed to amplify the polymorphic (TA)n repeat at 1174-base pair upstream of the human ER gene (10,11). The reaction was carried out in a final volume of 10 μl containing 100 ng of genomic DNA obtained from peripheral white blood cells, 5 pmol of each primer (5'-GACGCATGATAT-ACTTCACC-3' and 5'-GCAGAATCAAATATCCAGATG-3'), 400 μM of dGTP, dATP and dTTP, 40 μM of dCTP and 0.16 μl of [^{32}P]-dCTP (37 MBq/100 μl), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin and 1U Taq DNA polymerase (TAKARA SHUZO Co., Kyoto, Japan). Thirty PCR cycles (each for 2 min at 94 °C, 1 min at 58 °C, and 1 min at 74 °C) were performed. Electrophoresis was performed on 5% polyacrylamide gels containing 7M urea with control DNAs having known length followed by autoradiography. The

length of TA repeat in each amplified product was determined in comparison with sequence ladder of control DNAs.

Statistical analysis

Comparisons of the Z scores between the group of individuals possessing one allele of the genotype and the group not possessing that genotype were performed using non parametrical analysis (Student-Newman-Keuls Analysis). Differences in means were considered statistically significant for p values <0.05.

RESULTS

The primers for PCR were designed so that the PCR products contained upstream and downstream sequences of TA dinucleotide repeat (11) (Fig. 1). Using these primers in the reaction described in the methods, the size of PCR products ranged from 160 base pairs (containing 10 TA repeats with the 140 bp of amplified flanking sequences) to 194 bp (27 TA repeats). Eighteen alleles observed in this population were classified into genotype 'A' through 'R' according to the number of dinucleotide repeats they contained (Fig. 2). The frequency distribution of this polymorphism in all of the 274 subjects (548 chromosomes) was plotted in Fig. 2. The distribution in the Japanese women was similar to that observed in Caucasians (11), with peaks at 13 repeats (166 bp) and 22 repeats (184 bp). Correlations between the ER genotype and BMD Z scores or biochemical parameters were examined among 144 women about whom all of the clinical data were available. As a result, individuals possessing one allele of 'C' genotype (12 TA repeats) had significantly lower BMD Z scores of the spine and total body compared to individuals not possessing 'C' alleles (Fig. 3 a, b). We designated this allele (12 TA repeats) as a genotype C, because this size is the third smallest among all genotypes observed. In addition, concentrations of serum I-OC, urinary Pyr, and urinary Dpyr were significantly higher in the former group (Fig. 3 c, d,e). The background data of the group of genotype C and the other group is shown in Table 1. There were no significant differences between each group in age, weight and height. None of the study participants was homozygous for genotype C.

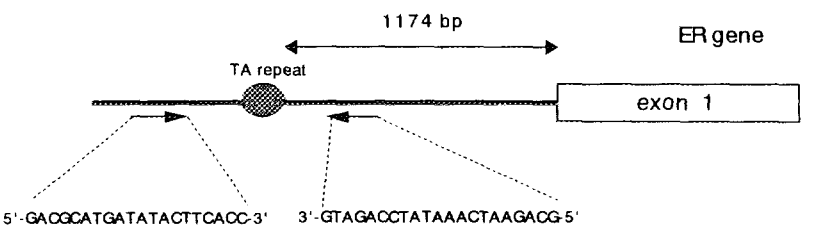

Fig. 1. The locus of TA repeat and PCR primers. Dinucleotide repeat located at 1174 bp upstream of exon 1. Each primer was designed to amplify the region including TA repeat.

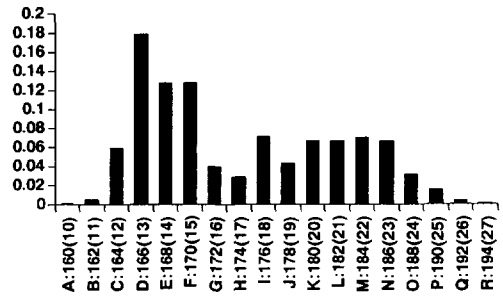

Fig. 2. Frequency distribution of dinucleotide repeat polymorphism. The frequency distribution of dinucleotide repeat polymorphism in all of the 274 subjects (548 chromosomes) was plotted. The PCR products ranged in length from 160 base pairs (10 repeats, genotype A) to 194 base pairs (27 repeats, genotype R).

DISCUSSION

In this study, the Japanese postmenopausal women who had an allele of genotype C showed lower BMD and elevated biochemical bone metabolic markers. Lower BMD in postmenopausal women can be considered as a result of abnormally rapid bone loss and/or lower peak bone mass (bone mass at the young adult). The bone metabolic markers which were significantly higher in the genotype C group represent accerelated bone resorption. These data suggest that the genotype C could affect BMD of postmenopausal women through accerelated bone loss. In other words, the genotype C at the microsatellite locus may be associated with some variation of the ER gene that cause the increased bone resorption after menopause. On the other hand, the study should be extended to know whether the genotype C also affects the peak bone mass.

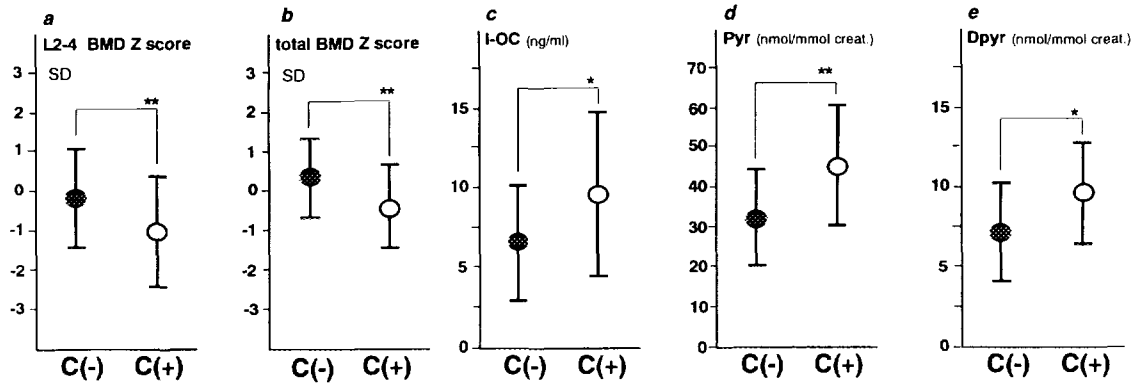

Fig. 3. Comparison of Z scores of bone mineral density (BMD) and biochemical bone metabolic markers between the group of individuals possessing one allele of genotype C (C+) and the group of individuals lacking genotype C (C-). a; spine BMD, b; total BMD, c; intact osteocalcin (I-OC), d; pyridinoline (Pyr), e; deoxypyridinoline (Dpyr) (*$p<0.05$, **$p<0.01$).

Table 1 Comparison of age, body weight, and height between the groups with or without genotype C

	C (-)	n	C (+)	n	p
Age (years)	64.8 ± 10	129	70 ± 13.9	15	NS
Weight (kg)	51.9 ± 8.4	129	50.9 ± 8.9	15	NS
Height (cm)	150.6 ± 6.4	129	148.3 ± 6	15	NS

C(-); the group of individuals not possessing genotype C.
C(+); the group of individuals possessing one allele of genotype C.
Data were analyzed using Student-Newman-Keuls analysis and expressed mean±SD.
NS: not significant.

Recently, a patient who inherited a disruptive point mutation in the ER gene (a cytosine-to-thymine transition at codon 157 of both alleles, resulting in a premature stop codon) was reported (12). The patient exhibited tall stature, incomplete epiphyseal closure, low bone density, and high bone turnover. Studies of "knockout" mice with a disruption in exon 2 of the ER gene were also reported (13). Bone densities in both female and male ER knockout mice were 20 to 25% lower than in wild-type mice (14). However mutations or variation of ER gene other than these disruptive mutation have not been reported in concern with bone metabolism or bone density. The results presented here in this report show the importance of further investigation for the variation of ER gene. We are now studying these possibilities using PCR-single strand conformational polymorphism and sequencing of ER gene in people who have low BMD.

Morrison *et al.* (15) reported that genetic variation at the vitamin D receptor locus accounts for up to 75% of the genetic basis of BMD in a selected Australian population. However, this association did not apply generally to other populations (16,17). Considering the pathogenesis of postmenopausal osteoporosis, analysis of possible roles of ER gene variations would be rationale to investigate the genetic background of this disease. We also suggest that the polymorphic microsatellite at the ER locus may become a useful marker for predicting future bone loss and to permit early therapeutic intervention in women at high risk for osteoporosis.

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