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Estrogen Receptor β Dinucleotide (CA) Repeat Polymorphism is Significantly Associated with Bone Mineral Density in Postmenopausal Women

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Abstract. Significant associations between the lengths of a highly polymorphic dinucleotide (CA) repeat located within the human estrogen receptor beta (ESR2) gene on chromosome 14, bone mineral density (BMD) and androgen levels have been reported previously in premenopausal women. We measured the size of this microsatellite repeat in 226 healthy women (60–98 years). After adjustment for age, body mass index, hormone replacement status, and other variables known to influence BMD, women with <25 CA repeats had significantly higher BMD measured in the total skeleton, lumbar spine, and femoral neck when compared with women having longer alleles. Women with shorter alleles also had higher circulating estrone and estradiol levels that approached statistical significance as compared with women harboring longer alleles after appropriate adjustments were performed in linear regression models. Women having both short and long CA repeats had BMD values in all regions of the skeleton that were midway between those found in women homozygous for longer or shorter repeat sizes. Because the ESR2 CA repeat size was neither associated with change in BMD nor serum levels of biochemical markers of bone turnover, it is likely that ESR2 CA repeat genotype is significantly linked to the attainment of peak bone mass in women.

Key words: Estrogen receptor beta — Estrogen receptor 2 (ESR2) — Polymorphism — Bone mineral density — Elderly women — Osteoporosis — Osteopenia

In 1998, Tsukamoto and coworkers [1] characterized a highly polymorphic dinucleotide (CA) microsatellite repeat (IVS5-3919) in the fifth intron of the human estrogen receptor beta (ESR2) gene residing within chromosome 14q22-q24 (Fig. 1). ESR2 is expressed in all bone cells, especially in the cancellous skeleton [2–5], and its mRNA level increases 20-fold in developing human osteoblasts [5]. A significantly higher Z-score lumbar-spine bone mineral density (BMD) was noted by Ogawa and coworkers [6] in postmenopausal Japanese women bearing at least one allele that was 26 repeats in length. Lau and colleagues [7] observed that premenopausal Chinese women bearing at least one ESR2 allele of 20 CA repeats had significantly higher BMD measured in the lumbar spine, total hip, and femoral neck, but these associations were not detected in postmenopausal women. Westberg and colleagues [8] reported that premenopausal Swedish women who had relatively smaller-sized ESR2 CA repeats had significantly higher circulating levels of total testosterone when compared with women with larger-sized repeats.

Dinucleotide microsatellite repeats have been mapped within introns or promoter regions of the human insulin–like growth factor-1 (IGF-1) [9], interleukin-6 (IL-6) [10], osteocalcin [11], calcium-sensing receptor [12], and calcitonin genes [13] and, with the exception of IGF-1, have been modestly associated with regional BMD measurements made on postmenopausal Japanese women [10–13]. CA microsatellite repeats are also located within promoter regions of matrix metalloproteinase [14] and type-I collagen α-2 genes [15], as well as in clones containing tumor necrosis factor-α [16]. Because these proteins play important roles in the development and maintenance of the skeleton, such polymorphisms may influence regional BMD and the rate of postmenopausal bone loss. Other significant candidate polymorphisms associated with BMD and osteoporosis have been reviewed elsewhere [17–19].

It is most likely that the interaction of several genes accounts for the wide between-individual differences ob-

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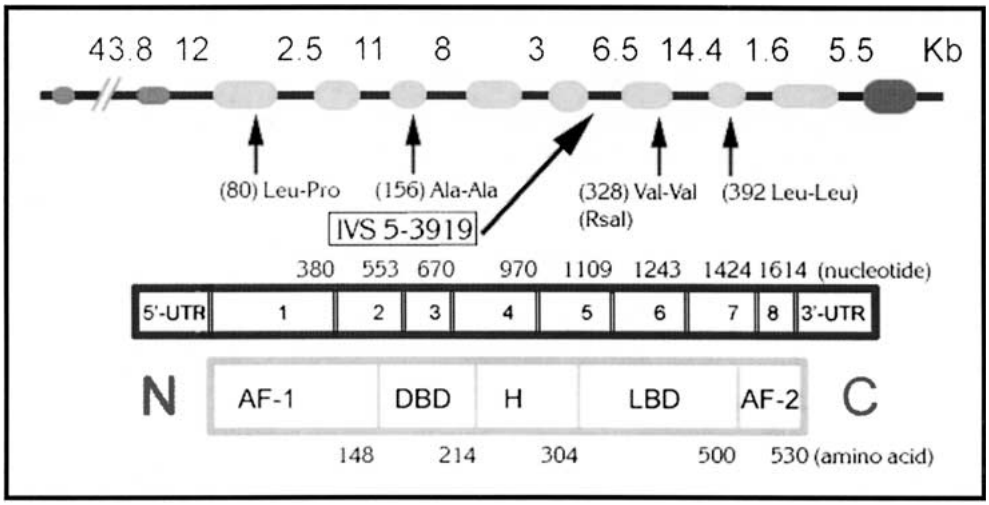

Fig. 1. Schematic of the ESR2 genetic locus (14q22-24) showing (top) the 8 exons (lighter shaded segments) and size of introns in kilobases. The two major transcription start sites are shown to the left, and an additional exon that may contribute amino acids to the *C*-terminal end of the protein is indicated to the right of the 8 exons. Locations of all the characterized coding region single-nucleotide polymorphisms are indicated below the locus map (small vertical arrows with the position of the corresponding amino acid). The residence of the microsatellite repeat polymorphism (IVS 5-3919) is indicated with a larger diagonal arrow. The middle diagram shows the mature ESR2 transcript with corresponding exons and nucleotide positions. The bottom diagram indicates the major domains of the ESR2 protein with corresponding amino acid positions. AF, transactivation domains; DBD, DNA-binding domain; H, hinge region; and LBD, ligand-binding domain.

served in densitometric measurements of the spine and hip [19]. Our preliminary efforts, however, were focused on the ESR2 CA microsatellite repeat, primarily because of the beneficial effects of hormone replacement therapy (HRT) that we observed previously in this study population [20]. For example, women who received 0.625 mg premarin/day over a 3 to 7 year period had significantly lower circulating levels of crosslinked *N* terminal telopeptides of type-I collagen (NTX), a selective index of osteoclast activity [21], and higher BMD measured at three anatomical sites as compared with those subjects not receiving HRT. Furthermore, the circulating estrone and estradiol levels were an important determinant of not only the circulating levels of biochemical markers of bone metabolism, but also was significantly associated with regional BMD measurements and changes in BMD that we recorded over a 3-year period [22].

Because the result of multiple regression models indicated that the circulating level of estrogens was a critical determinant of BMD in elderly women, we surmised that polymorphisms within this locus might influence the expression or function of ESR2, a key mediator of estrogen-signaling in bone cells [2–5], and, therefore, may influence the status of the skeleton in elderly women. Such an analysis potentially could explain, in part, the heterogeneity in BMD and bone markers we noted across the cohort of women receiving HRT. Furthermore, the ligand-binding domain of ESR2 differs from that of the classical estrogen receptor ER-α [23], ESR2 may, therefore, be a sensitive and selective target for novel therapeutic agents aimed at preserving bone mass.

The purpose of the present investigation was, therefore, to determine if the ESR2 microsatellite genotype influences regional and total skeletal BMD, the rate of change in BMD over a 5–7 year period, biochemical markers of bone metabolism, and hormone status in 226 healthy female participants in the New Mexico Aging Process Study.

Materials and Methods

Study Population

Two hundred and twenty-six ambulatory, community-dwelling female volunteers (age 60–98 years) were recruited, after having provided written informed consent, from the New Mexico Aging Process Study (NMAPS) at the University of New Mexico Health Sciences Center. Excluded from the study were individuals with recent myocardial infarction; uncontrolled hypertension; recently diagnosed malignancy (other than skin); chronic obstructive pulmonary disease; as well as those who were immobile or who were receiving chemotherapeutic, cardiac, respiratory, glucocorticoid, or antipsychotic medications. Each subject underwent a thorough physical examination and was given a detailed questionnaire. BMD was measured and blood samples were drawn both at entry into the study in 1993 and once a year thereafter and continuing presently.

The NMAPS cohort is not strictly population-based; it is composed of volunteers and the entry criteria exclude those with serious diseases. Thus, our results may or may not be generalizable to the broader population. The representativeness of the NMAPS can be judged by comparison with the population-based study we conducted between 1993 and 1996 in the Albuquerque area, the New Mexico Elder Health Survey (NMEHS). This survey was designed to randomly sample community-dwelling, ambulatory elders, but excluded those institutionalized in nursing homes that would have more serious morbidity. In 1993, 17% of active NMAPS participants had a previous history of coronary heart or cardiovascular

disease (CHD/CVD), 32% had hypertension, 72% had osteoarthritis, and 15% had a previous diagnosis of some form of cancer. The corresponding disease prevalence among the NMEHS was 18% CHD/CVD, 32% hypertension, 66% arthritis, and 19% cancer. Thus, the prevalence of these types of major morbidities does not differ significantly from that found in a larger and more general population.

The participants were caucasian, with 7% claiming Hispanic ancestry. No differences in BMD, rate of fracture, falls, prevalence of osteoporosis, circulating levels of biochemical markers of bone metabolism, or ESR2 microsatellite genotype frequency were detected within the cohort claiming Hispanic ancestry in the present or previous studies [20, 22].

Seventy-five women reported receiving 0.625 mg of Premarin per day with 2.5 mg of medroxyprogesterone acetate. Self-reported HRT was confirmed by the measurement of serum estradiol and estrone levels. Thirty-three subjects were receiving 10 mg alendronate daily, and nine women reported 200 U daily nasal calcitonin use in addition to alendronate therapy. A breakdown of women receiving any single, or combination of medication is categorized in Table 1, along with the corresponding ESR2 microsatellite polymorphism allele frequencies within these cohorts, for which there was no statistically significant difference. The percentage of women receiving HRT (75/226, 33%) for three consecutive years after menopause in our population was identical to that determined for 1015 women living in Denmark in 1997 [24]. Ninety-eight women had a daily supplementation of ≤ 1.0 g of calcium carbonate for at least two of the study years, and most of the other subjects were taking 400 to 800 mg of calcium carbonate per day. Accordingly, all multiple regression analyses were adjusted for the effect of HRT, bisphosphonates, and nasal calcitonin, as well as for calcium intake. All procedures were in accordance with the Helsinki Declaration, and the study was approved by the University of New Mexico School of Medicine Human Research Review Committee.

Table 1. Medications received by the study population

	Medications	No medications
Total number	97	129
HRT alone (*n*)	64	
Alendronate alone (*n*)	16	
HRT + alendronate (*n*)	8	
Alendronate + nasal calcitonin (*n*)	6	
HRT + alendronate + nasal calcitonin (*n*)	3	
Genotype frequency (%)		
SS	50	46
SL	24	22
LL	26	32

Characteristics and allele frequency within cohorts of the study population showing number of subjects receiving and not receiving bone-preserving medications (hormone replacement therapy [HRT], alendronate, and nasal calcitonin). Differences in genotype frequencies among those receiving and not receiving medications are not statistically significant
LL, 2 copies of a risk allele; SL, 1 copy of a risk allele; SS, 0 copies of a risk allele

Bone Mineral Density

BMD was quantified by using whole body, femoral neck, and lumbar spine dual emission X-ray absorptiometry (Lunar DPX, System no. 6647, software version 3.6z; Lunar Radiation Corp, Madison, WI, USA) on each subject initially in 1993 and subsequently once a year thereafter utilizing the same instrument. All scans were performed by a licensed radiologic technician. The manufacturer's recommendations for calibration of the instrument were closely followed. The coefficients of variation for daily quality control for the large, medium, and small bone mineral standards were 0.40%, 0.33%, and 0.58%, respectively. Subjects assumed a supine position with arms at their sides, and extreme care was taken to ensure that participant positioning was consistent. A medium-speed mode was used for whole body scans of all subjects, and the scan time was 20 to 30 minutes depending on body size. The femoral neck and lumbar spine regions were scanned for 6 to 8 minutes for each site. Estimation of lumbar spine BMD excluded regions where compression fractures or degenerative changes were evident.

Ascertainment of ESR2 Genotype

Polymerase chain reaction (PCR) amplification of the ESR2 CA repeat polymorphism (Fig. 1) was performed as previously described [6], and in duplicate, utilizing 100 to 500 ng of genomic template in a final reaction volume of 50 μL containing 10 mM Tris-HCl (pH = 8.3), 1.5 mM $MgCl_2$, 200 mM dNTPs, 200 μM 6-FAM labeled forward primer (5′-GGTAAACCATGGTCTGTACC-3′), unlabeled reverse primer (5′-AACAAAATGTTGAATGAGTGGG-3′) and 0.2 U Taq polymerase (Fisher Scientific, Pittsburgh, PA, USA). Thirty-four PCR cycles at 94°C, 62°C, and 72°C each for 1 minute were performed. A total of 1 μL of amplified DNA was then denatured in 10 μL formamide with 0.5 μL TAMRA-labeled size standards for 10 minutes at 90°C, and was injected into an ABI Prism 310 (Applied Biosystems, Foster City, CA, USA) for sizing analysis. At least two separate internal quality controls that consisted of genomic DNA isolated from laboratory volunteers were utilized in each set of PCR products performed and assayed. The CA repeat sizes of the controls were consistently reproduced without deviation or error throughout the period of study. Sequencing reactions were performed on 10 of the subjects to verify the correct association of the CA repeat number with the deduced molecular mass of the PCR product, originally reported by Ogawa [6]. ESR2 genotype was classified utilizing criteria described by Westberg [8], whereby alleles were defined as being short (S) or long (L), using median values as cutoff levels determined for our study population, namely 24 CA repeats for the smaller-sized allele and 25 repeats for the larger-sized allele.

Hormones and Biochemical Markers of Bone Turnover

Bone specific alkaline phosphatase (bAP), (NTx), and extension propeptides of type I procollagen (PINP) were measured in the sera of all participants in 1993, 1996, and 1999, by using radioimmunoassay and enzyme-linked immunosorbent assay (ELISA) kits purchased from the Quidel Corporation (San Diego, CA, USA), Ostex International (Seattle, WA, USA) and Diasorin (Stillwater, MN, USA) respectively. Estradiol levels were measured in duplicate in the sera of women who reported HRT utilizing Estradiol-6® kits (Diagnostic Products Corporation, Los Angeles, CA, USA) that are optimized for specificity for 17-β-estradiol in the context of interfering steroids such as equilin and equilenin derived from Premarin®. For those women not receiving HRT, estradiol was measured using the DPC Estradiol Double Antibody® radioimmunoassay kit that is optimized for higher sensitivity. Serum levels of estrone and sex hormone–binding globulin were measured utilizing radioimmunoassay kits purchased from ICN Biomedicals (Costa Mesa, CA, USA) and Diagnostic Systems Laboratories Inc. (Webster, TX, USA), respectively.

Statistical Analysis

Least squares multiple regression modeling was performed with the JMP Statistical Discovery Software (SAS Institute,

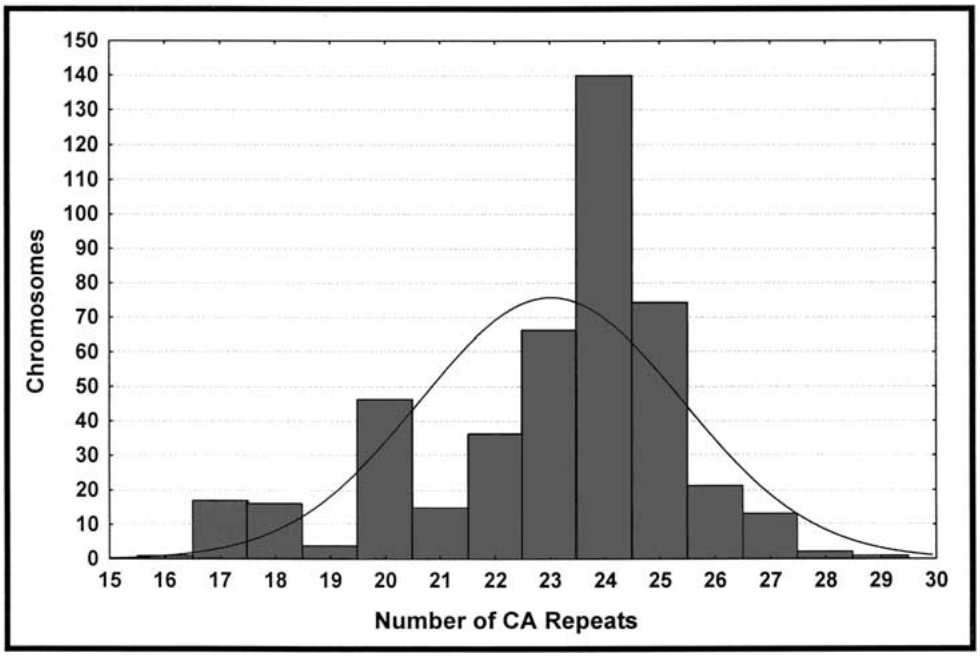

Fig. 2. Allele distribution histogram showing the frequency of the ESR2 CA repeat size in a population of 226 healthy, community-dwelling, nonrelated postmenopausal women living in Albuquerque, New Mexico. Heterozygosity index = 0.83.

Cary, NC, USA) with use of BMD measured in the total skeleton, lumbar spine, and femoral neck as dependent variables and ESR2 genotype as the independent variable. ESR2 genotype was categorically defined as SS (0 copies of a risk allele), SL (1 copy), or LL (2 copies) using JMP's "fit model" multiple linear regression menu. The *P*-value was calculated for the number of copies of the risk allele and tested the statistical significance of the trend. Age, body mass index, hormone replacement status, estradiol levels, nasal calcitonin, bisphosphonate usage, and intake of 1.0 g or more of daily calcium carbonate were included in the models as confounding covariates. Similarly, estradiol and estrone levels were used as dependent variables in multiple regression analysis with ESR2 genotype as the categorical independent variable, after adjustment for age, body mass index, hormone replacement status, and circulating levels of sex hormone–binding globulin.

Results

ESR2 CA Dinucleotide Repeat Length and Sequence

The frequency and distribution of the ESR2 microsatellite repeat size in our population is shown in Figure 2. The heterozygosity index calculated for our population was 0.83. By using the median values for the smaller-sized (24 repeats) and larger-sized (25 repeats) allele as cutoff values, as was performed in a study of 241 premenopausal women by Westberg et al. [8], 108 subjects were classified as having two short alleles (SS), 67 had two long alleles (LL), and 51 were defined as having a short and long allele (SL). The DNA sequence generated using PCR products amplified from 10 homozygous subjects spanning the range of 20 to 25 repeats confirmed the association of the number of CA repeats with the deduced molecular mass of the PCR product reported originally by Ogawa [6] and matched the published human chromosome 14 sequence in the flanking regions of the polymorphism (Fig. 1).

Bone Mineral Density

Table 2 summarizes the least-squares-means and results of multiple regression modeling using regional and total skeletal BMD measurements compiled between 1999 and 2000 as dependent variables. Adjustment of the multiple linear regression models included the continuous variables of age and body mass index, and the categorical variables of hormone replacement status (0.625 mg Premarin daily in 75 subjects), bisphosphonate, or nasal calcitonin use (33 and 9 subjects, respectively) as significant confounding covariates. Calcium or vitamin D intake was not significantly associated with BMD in this population, especially considering that almost every subject was receiving some form of calcium and vitamin D supplementation. Women having two short ESR2 CA dinucleotide repeats (SS) had significantly increased BMD at all skeletal regions as compared with women having two long (LL) alleles (Table 2, Fig. 3, 4). Furthermore, women having the mixed short and long (SL) genotype had BMD values at all skeletal regions that were intermediate in value between the extremes of the SS and LL genotypes. The stratification of BMD as highest in SS, intermediate in SL, and lowest in LL genotypes was statistically significant and consistently reproduced in any year of data analyzed from 1993 until the present. Adjustment using estrone or estradiol levels as a covariate did not diminish the significance of the differences observed in BMD at any skeletal region.

By using the endpoints of BMD and bone turnover markers, we could detect no significant interaction of the ESR2 microsatellite genotype with use of HRT. Consequently ESR2 genotype did not influence the efficacy

Table 2. Results of multiple linear regression analysis showing significant association of total and regional bone mineral density measurements with ESR2 genotype

	TBMD (g/cm^2)	SBMD (g/cm^2)	FBMD (g/cm^2)
SS (n = 108)	1.022 ± 0.015	1.049 ± 0.036	0.752 ± 0.018
SL (n = 51)	1.004 ± 0.015	0.999 ± 0.038	0.735 ± 0.018
LL (n = 67)	0.983 ± 0.017	0.959 ± 0.042	0.703 ± 0.021
P-value	0.02	0.04	0.02

Least square means (±standard error) of total body bone mineral density (TBMD), lumbar spine bone mineral density (SBMD), and femoral neck bone mineral density (FBMD) measurements made by dual X-ray absorptiometry showing significant effect of the estrogen receptor β CA repeat polymorphism length where SS = women having two short alleles, LL = women having two longer CA repeats, and SL = women having both a short and long allele as defined previously. BMD measurements are adjusted by regression analysis for age, body mass index, hormone replacement status, and use of bisphosphonates or nasal calcitonin as confounding covariates

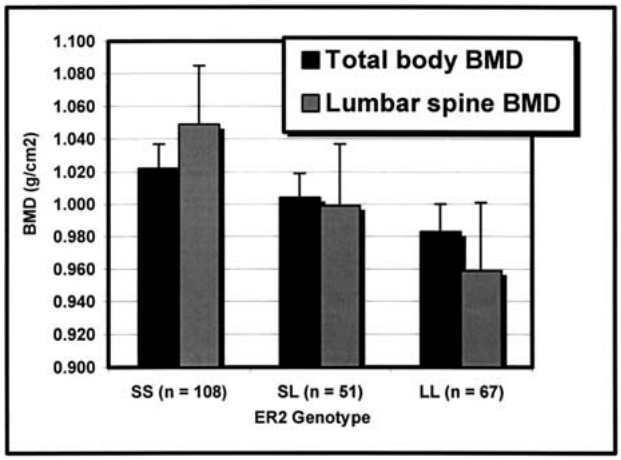

Fig. 3. Least squares means (± standard error) of total body and lumbar spine bone mineral density measured in 1999 as a function of ESR2 genotype. SS = women having two short alleles, LL = women having two longer CA repeats, and SL = women having both a short and long allele, as defined previously. Least squares means are adjusted in regression analysis for age, body mass index, hormone replacement status, and use of bisphosphonates or nasal calcitonin as confounding covariates. BMD, bone mineral density.

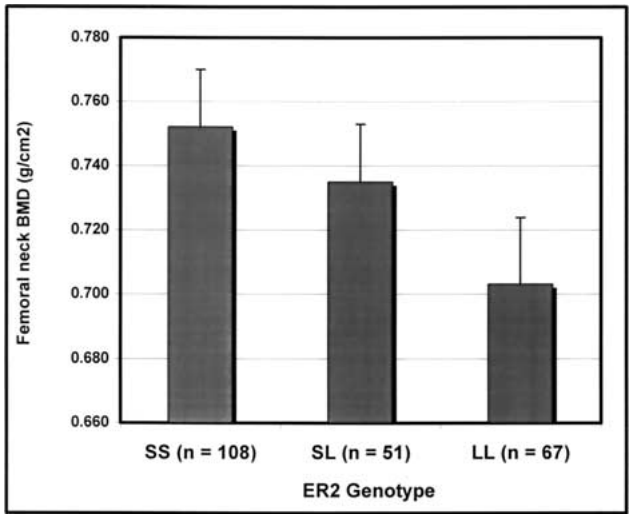

Fig. 4. Least square means (±standard error) of femoral neck bone mineral density (BMD) measured in 1999 as a function of ESR2 genotype. SS = women having two short alleles, LL = women having two longer CA repeats, and SL = women having both a short and long allele, as defined previously. Least square means are adjusted in regression analysis for age, body mass index, hormone replacement status, and use of bisphosphonates or nasal calcitonin as confounding covariates.

of hormone replacement status within this cohort of our study population.

Change in Bone Mineral Density and Biochemical Markers

To identify factors that account for the variability in the 5 to 7 year change in BMD we monitored in our study population, and the wide observed between-individual differences in circulating levels of biochemical markers of bone metabolism, we performed multiple regression modeling using the change in whole skeleton, spine, and hip BMD, or the serum NTx, PINP, and bAP levels as dependent variables. As anticipated, after adjustment for age and BMI, the 5 to 7 year loss in BMD, expressed as the least squares mean percentage of baseline, was significantly lower in the 97 women who were receiving hormone replacement or antiresorptive therapies such as bisphoshonates or nasal calcitonin, compared with the 129 women who were not receiving any form of medication aimed at minimizing bone loss (data not shown). When controlling for age, BMI, and use of HRT, bisphosphonates, or nasal calcitonin, we could not detect any significant effect of the ESR2 CA repeat polymorphism length on influencing the longitudinal change in BMD or circulating levels of biochemical markers of bone metabolism in this study population (Table 3).

Circulating Estrogen Levels

As shown in Table 4, measurement of serum estrone levels performed in 1996 indicated that women with the SS genotype had significantly higher values; women harboring the LL alleles had the lowest levels, and

Table 3. ESR2 genotype, biochemical markers of bone metabolism and 5–7 year change in bone mineral density

	ΔTBMD (%)	ΔSBMD (%)	ΔFBMD (%)	NTx (nmoles/L)	bAP (U/L)	PINP (μg/L)
SS (n = 108)	0.88 ± 0.49	4.02 ± 1.45	0.23 ± 1.37	12.8 ± 0.7	20.4 ± 1.1	24.1 ± 5.2
SL (n = 51)	0.49 ± 0.62	1.94 ± 1.83	1.72 ± 1.73	12.7 ± 0.9	22.9 ± 1.4	28.0 ± 6.8
LL (n = 67)	0.64 ± 0.58	3.68 ± 1.69	1.46 ± 1.60	13.0 ± 0.9	21.5 ± 1.4	33.7 ± 6.3
P-value	0.80	0.48	0.23	0.96	0.18	0.37

Least squares means (± standard error) of the 5–7 year percentage change from baseline in total body bone mineral density (αTBMD), lumbar spine bone mineral density (αSBMD) and femoral neck bone mineral density (αFBMD) measurements made by dual X-ray absorptiometry and serum levels of *N*-terminal crosslinked telopeptides of type-I collagen (NTx), bone-specific alkaline phosphatase (bAP), and extension propeptides of type-I procollagen (PINP) showing no significant effect of the estrogen receptor β CA repeat polymorphism length. SS = women having two short alleles, LL = women having two longer CA repeats, and SL = women having both a short and long allele as defined previously. Means for ΔBMD and biochemical markers are adjusted in regression analysis for age, body mass index, hormone replacement status, and use of bisphosphonates or nasal calcitonin as confounding covariates

Table 4. Circulating estrogen levels and ESR2 genotype

	1996 Estrone (pg/mL)	2000 Estrone (pg/mL)	2000 Estradiol (pg/mL)
SS (n = 108)	69.2 ± 8.7	82.6 ± 8.2	35.2 ± 2.7
SL (n = 51)	48.1 ± 10.0	80.8 ± 11.2	32.4 ± 3.7
LL (n = 67)	24.9 ± 10.6	63.0 ± 11.9	25.8 ± 3.9
P-value	0.006	0.37	0.13

Least squares means (±standard error) of serum estrone (measured in 1996 and 2000) and 17-β estradiol levels measured in 2000 showing the effect of the estrogen receptor β CA repeat polymorphism length where SS = women having two short alleles, LL = women having two longer CA repeats, and SL = women having both a short and long allele as defined previously. Hormone measurements are adjusted in regression analysis for age, body mass index, hormone replacement status, and levels of sex hormone binding globulin as confounding covariates

women with the SL genotype had a circulating estrone concentration that was intermediate between the SL and LL genotypes, after adjustment for age, BMI, hormone replacement status, and circulating levels of sex hormone–binding globulin. Similar analysis of serum 17-β estradiol and estrone levels in the year 2000, revealed similar trends that were not statistically significant at a 95% confidence level. We did not detect a significant interaction of the ESR2 genotype and estrogen status within the cohort of women receiving HRT.

Discussion

The most important finding of this study is the significant association we detected of the ESR2 microsatellite polymorphism with selected endpoints of skeletal and endocrine status in postmenopausal women. A dosage effect of the ESR2 CA repeat alleles was suggested by the observation that women with the mixed SL genotype had estrogen levels and BMD values measured in all regions of the skeleton that were midway between the extremes of the SS and LL genotypes. Moreover, the typical femoral neck and lumbar spine BMD values measured in women having the LL genotype were indicative of an osteopenic cohort.

We defined the ESR2 genotype by utilizing an approach published by Westberg and colleagues [8] using the median size of each allele as a cutoff value. Such an *ad hoc* classification by nature is problematic. Our findings support previous associations of this polymorphism with the BMD phenotype of Asian women [6, 7] and the androgen status of Swedish women [8].

It is interesting to note that the shape of the histogram showing the frequency of the CA repeat alleles in our New Mexican study population (Fig. 2) does not fit a normally distributed curve and is essentially identical to that which was reported for approximately the same number of premenopausal Swedish women [8]. Although the ESR2 allele frequency histogram appears to have a bimodal distribution in postmenopausal Japanese women [6], the shape and amplitude of this curve differs substantially from that of populations living in Sweden or New Mexico. The similarity of the bimodal New Mexican (Fig. 2) and Swedish allele distributions [8], especially toward the lower limit of CA repeat size, is remarkable, as it underscores a strong similarity across divergent European populations with respect to this polymorphism.

Our results differ from those of Ogawa [6] and Lau [7] who associated an allele of 26 or 20 CA repeats, respectively, with a higher BMD in healthy Japanese postmenopausal and Chinese premenopausal women, and, additionally with data acquired from a larger North American study population [25]. Such discrepancies are most likely explained by the different statis-

tical approach utilized (dichotomous genotype versus individual allele analysis) and variability in allele and BMD frequencies among Asian and European populations [6–8]. The presence of significant ethnic admixture in a larger North American sample may have confounded such a population-based association study [25–28]. Our results are in agreement, however, with the former investigations [6, 7, 25] with respect to the importance of this locus in influencing BMD. The replication of results across association studies in different populations is important, as this study design is susceptible to producing false positive results.

The ESR2 CA repeat polymorphism length had no influence on circulating levels of biochemical markers of bone metabolism, nor on the 5 to 7 year change in regional and total BMD we calculated, suggesting that this polymorphism is more strongly associated with the attainment of peak bone mass than with the decline in BMD after menopause. Due to the advanced age of our study population, however, we were not able to monitor bone loss in the critical years immediately following menopause. Future studies of the ESR2 CA repeat polymorphism are, therefore, warranted in larger populations of pre- and perimenopausal women.

Precisely how an intronic microsatellite repeat influences the expression or function of ESR2 or other genes in which such polymorphisms are under active investigation [29–33] is currently under debate. Gebhardt and colleagues [33] demonstrated that a dinucleotide repeat polymorphism located within the first intron modulated the *in vitro* transcription of the human epidermal growth factor receptor gene. An intronic dinucleotide repeat within the C-reactive protein locus was correlated with its baseline expression level [34], or local chromatin structure, and attachment to the nuclear matrix may be modulated by microsatellites [35, 36]. A recent study undertaken in Finland indicated that breed of the domestic canine can unambiguously be assigned by analyzing multiple microsatellite polymorphisms [37]. Alternatively, an attractive hypothesis is that this polymorphism influences the differential expression of the numerous splice variants of ESR2.

The principle limitation of our investigation is that our results can be generalized to only healthy elderly women. There is also the possibility, as in all association studies, that covert stratification within our sample could have produced spurious results. Despite such inherent pitfalls of population association, our preliminary study of this polymorphism taken with data from other investigations [6–8, 25] demonstrates the necessity for undertaking a high-resolution haplotype analysis using larger populations that are more inclusive of men and women at risk for osteoporosis. Such an approach will reveal if the ESR2 microsatellite repeat is in linkage with the only nonsynonymous single nucleotide polymorphism (SNP) characterized at the date of submission of this data in the entire ESR2 locus (nt317 Leu $\rightarrow$ Pro), or with one of a panel, selected from the 177 other SNPs residing within the ESR2 locus on the basis of position and likelihood to influence alternative splicing. We are also currently investigating the possibility that microsatellite genotype affects the levels or induction of ESR2 and perhaps influences the differential expression of the various ESR2 isoforms in human peripheral blood mononuclear cells.

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