BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **248,** 190–195 (1998)
ARTICLE NO. RC988880

Polymorphisms of the Calcitonin Receptor Gene Are Associated with Bone Mineral Density in Postmenopausal Italian Women

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Received May 22, 1998

Recognition of a major genetic component in bone mass determination represented the basis for studies aiming to the identification of underlying major and minor genes. Bone mineral density (BMD) represents the continuous trait to be quantified in order to evaluate segregation of candidate genes with risk of osteoporosis. Polymorphisms at the vitamin D receptor (VDR), estrogen receptor, (ER), collagen type I, and interleukin 6 (IL6) gene loci have been correlated to BMD. However, in a polygenic disorder, such as osteoporosis, the number of genes expected to influence BMD is very large. In the present study we examined the presence of restriction fragment length polymorphisms (RFLPs) for the calcitonin receptor (CTR) gene in postmenopausal women. We identified a polymorphic (Tt) site at the CTR gene locus using the Taq I restriction fragment enzyme. Three genotypes were observed, whose Tt was the most frequent in our population (49.7%). In addition, Ancova analysis and Tukey's test showed that women with tt genotype had significantly lower lumbar BMD in comparison with Tt genotype (Tukey's test: p = 0.005). In conclusion, evidence of RFLPs at the CTR gene locus in Caucasian postmenopausal women of Italian origin made it possible to identify the involvement of another gene, the CTR gene, in the determination of bone mass. © 1998 Academic Press

Osteoporosis is recognized to be one of the most important disorders of aging. Increasing interest in osteoporosis has called attention to many factors that in aggregate speak to a disease of multifactorial etiology. It is also well known that peak bone mass is primarily under genetic control (1). Twin studies have shown strong genetic effects on bone mineral density at both peripheral and axial bone sites. Using this model Pocock et al. observed that genetic effects at the lumbar spine and femoral neck explain up to 80% of the population variation in BMD (1). Because of the increasing evidence that genes are important in the determination of peak bone mass, studies were undertaken in order to test relationships between DNA polymorphisms of candidate genes and osteoporosis-related phenotypes. Haplotype analysis based on 3-restriction fragment length polymorphisms at the VDR gene locus offered the possibility to discriminate individuals at high and very low risk of osteoporosis (2-4). Successive studies, however, did not support these findings (5-8). Various suggestions have been made to account for these discrepancies, such as racial and environmental differences, inadequate sample size and confounding factors. Discrepancy among studies can also be explained on the basis of the quantitative polygenic nature of this disorder, where the effect of a given gene can easily be modified by epistatic and/or pleiotropic effects of other genes. Indeed, the introduction of another variable, the ER genotype, in the analysis of the VDR genetic determination of bone mineral density showed a clear interaction between the two loci in determining BMD (9). Other polymorphic genes could similarly contribute to bone mass determination via reciprocal interactions.

One of the hormones involved in bone metabolism is calcitonin (CT), a polypeptide hormone secreted by parafollicular cells of the thyroid gland (10), able to inhibit osteoclastic bone resorption and to stimulate urinary calcium excretion (11). The human CT receptor (CTR) was cloned from an ovarian carcinoma cell line (BIN-67) (12) and recognized to be a prototypic member of a distinct family of G-protein-coupled receptors with

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0006-291X/98 $25.00

TABLE I

Characteristics of the Population

	Normal	Osteoporotic	Total
Number	111	90	201
Age (yrs)	58.3 ± 0.5	59.1 ± 0.3	58.7 ± 0.4
YSM	10.1 ± 0.5	12.3 ± 0.3	11.2 ± 0.7
Height (cm)	163 ± 0.8	158.5 ± 0.6	162.5 ± 0.6
Weight (Kg)	62.5 ± 1.3	63.7 ± 1.1	63.1 ± 0.5
LS-BMD (g/cm^2)	0.919 ± 0.1	0.733 ± 1.66	0.840 ± 0.15
F. Neck BMD (g/cm^2)	0.693 ± 0.4	0.607 ± 0.7	0.650 ± 0.9
Ca-intake (mg/day)	565 ± 133	578 ± 140	571.5 ± 103
SPO score	0.67 ± 0.03	0.65 ± 0.05	0.66 ± 0.04
FOA score	0.60 ± 0.02	0.57 ± 0.03	0.57 ± 0.01

seven spanning domains (13). Different isoforms of CTR resulting from alternative splicing of the gene have been described in various animal species with differential tissue expression transcripts (14, 15) and different signaling properties (16). Two different isoforms have been described in human giant cell tumor of bone (17). The first isoform, designed as GC-10, differs from the previously described ovarian-human CTR gene (12) in the 5′-region in that it lacks a 71-bp segment, while being almost identical in the 3′-region (17). The second human GCT-CTR cDNA variant, indicated as GC-2, lacks this 71-bp 5′ insert but also 48 nucleotides encoding part of the first intracellular domain (17). It is likely that differential expression of CTR isoforms is a mechanism of regulation of biological responses to CT. Shift in the predominant CTR isoform(s) could also explain the variable responsiveness to CT in patients with high turnover metabolic bone diseases (17).

In the present study we describe a previously unrecognized RFLP for the human CTR gene and the distribution of its allelic variants in a population of Caucasian postmenopausal women of Italian descent, in order to evaluated the correlation of CTR genotypes with lumbar and femoral neck BMD. Three major genotypes were identified with different distribution in normal and osteoporotic subjects.

SUBJECTS AND METHODS

Subjects. Patients were postmenopausal women (n = 201) with an age range of 47-76 years and a mean (±SEM) age of 58.7 ± 0.4 years. Patients were selected at the metabolic bone diseases outpatient Clinics in Florence and Siena among 1,500 women who underwent clinical examination for osteoporotic risk evaluation in 1995 (9). Women known to be affect by metabolic bone diseases other than primary osteoporosis, such as primary hyperparathyroidism, hyperthyroidism, diabetes, cirrhosis, and kidney failure were excluded. According to WHO criteria (18) women were divided in osteoporotic (n = 90) and normal (n = 111). For all women, a detailed medical history was obtained and dietary calcium intake was assessed by a sequential self-questionnaire including foods that account for the majority of calcium in the diet. A large database has been completed for these women. Table I depicts the general features of the population. Women were requested to give informed consent for genetic evaluation.

Bone densitometry and X-ray examination. Lumbar BMD (L2-L4) was measured by dual-energy X-ray absorptiometry (DEXA; Hologic QDR 1000/Walthom, MA) in all 201 women, with coefficients of variation of 0.5% *in vitro* and 0.9% *in vivo*. BMD at the upper femur (neck, Ward's triangle, greater trochanter) was measured by DEXA with coefficient of variation of 0.6 % *in vitro* and 1.0% *in vivo*. Cross calibration on the precision of measurements between the two Centers of Florence and Siena was previously performed (9) and a correction factor was not considered necessary.

In addition, patients underwent a lateral lumbar spine X-ray examination in order to be scored for spinal osteophytosis (SPO) according to Orwoll (19) and for facet joint osteoarthritis (FOA) on a four point scale (0=none, 1=mild, 2=moderate, 3=severe). Vascular calcifications were not evaluated for their reported limited impact on spinal density measurement (20).

Genotyping. Genomic DNA was isolated from EDTA blood samples by a standard phenol-chloroform extraction procedure and 8 μg of DNA were digested for 18 hrs in a volume of 50 μl using various restriction endonucleases: Apa I, Msp I, Xba I, Pvu II, Bsm I, and Taq I (Boehringer, Mannheim, Germany) at temperatures recommended by the manufacturer. The digested DNA was fractionated using 0.7-1% agarose gel eletrophoresis and transferred to nylon-based filters (gene Screen Plus, NEN Research Products, Boston, MA) by standard technique (21). Filter membranes were prehybridized, then hybridized with the ^{32}P-labelled probe for 18 h at 65°C, washed and autoradiographed for 24-48 h at −80°C. The probe used to identify RFLPs was a cDNA for the human ovarian CTR generously made available by A.H. Gorn (12). The probe was radiolabelled with [α-^{32}P] dCTP using a random priming labeling kit (Boehring Mannheim, Germany).

Statistical analysis. The frequency distribution of CTR genotypes in normal and osteoporotic groups was compared using standard Chi-squared test. Differences in anthropometric characteristics, spinal and femoral BMD among the different CTR genotypes were calculated using analysis of variance (ANOVA). Similar comparisons were performed after adjusting mean BMD values for potential confounding factors such as age, height, weight, and years since menopause (YSM) using analysis of covariance (ANCOVA). Tukey's test was used to compare the genotypes. Data were expressed as mean (±SEM), with $p < 0.05$ accepted as the value of significance. Statistical analysis was performed using STATISTICA program. The expected frequency for genotype for the CTR polymorphism was determined using a method previously described by Li (22).

RESULTS

A previously unrecognized RFLP at the human CTR gene was detected by the restriction endonuclease Taq I. No variant bands were recognized with MspI, Bsm I, Apa I, Pvu II, and Xba I enzymes. Digestion with Taq I generated 10 bands: 2 variant bands (respectively of 7 and 5 kb) and 8 invariant bands. The different alleles were recognized and designated Tt (heterozygous), TT and tt, where uppercase letter (T) indicates the absence and lowercase letter (t) the presence of the Taq I restriction site. The Southern blot-banding pattern for this enzyme is shown in Fig. 1. The Mendelian nature of the RFLPs was verified by family studies (data not shown). Three genotypes could be recognized by Taq I enzyme digestion (Fig. 1). Subjects in the three genotypes resulted well matched for age, height and

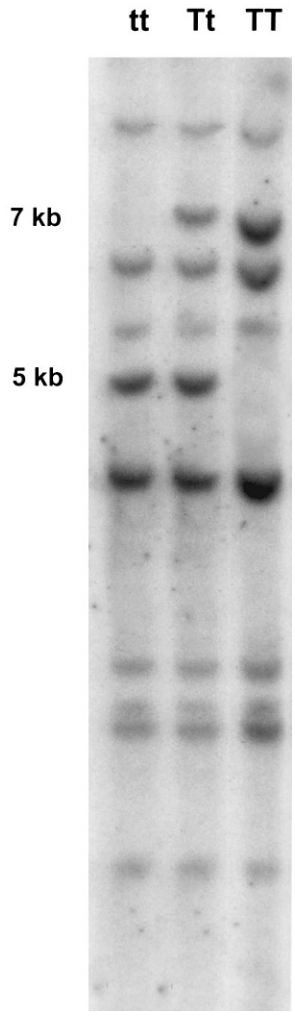

FIG. 1. RFLPs of CTR gene identified by Taq I enzyme. Blot banding pattern for Taq I enzyme is shown. Two variant bands were individuated and indicated as T(7 Kd), t (5 Kd). The three identified genotypes are indicated on the top.

weight. The allelic distribution of the CTR polymorphism is shown in Table II. In the present population the relative distribution of CTR genotypes followed the Hardy-Weinberg equilibrium (22).

The frequency expressed in percent of these RFLPs in our population and the differences in the distribution of the three genotypes are shown in Table III. Of the 201 analyzed women 100 (49.7%) had the Tt genotype, 79 (39.3%) had tt genotype and 22 (10.9%) had TT genotype. Applying the standard Chi-squared test we observed significant differences in the distribution of the genotypes in our population, as the Tt genotype was more represented in normal women in comparison with ostoporotic women, with a frequency of 57.8% vs.40%. Conversely, the tt genotype was more represented in osteoporotic women with a frequency of 45.5% vs. 34.2% ($\chi 2$ test=6.5 p=0.03). When we applied the variance test (ANOVA) to evaluate lumbar and femoral neck BMD differences among the three genotypes significant differences could be observed in mean BMD at lumbar spine (p=0.005) but not at the upper femur (p=0.07). Similar results were obtained applying analysis of covariance (ANCOVA) used to adjust mean BMD values for potential confounding factors such as age, height, weight, years since menopause (YSM), FOA, and SPO. We observed that in our population YSM, age, height and weight, were not determinant for bone mass both at the femoral neck and lumbar sites and did not change the results of variance analysis (data not shown). Similarly, FOA and SPO did not change the results both at the femural and lumbar spine (data not shown). Using Tukey's test we could detect that women with tt genotype had significant lower lumbar BMD in comparison with Tt (p=0.005) genotype (Fig.2).

ANOVA and ANCOVA analysis did not reveal any statistically significant difference among genotypes for femoral neck BMD.

TABLE II

Allelic Distribution of the T/t Polymorphism at the CTR Gene in the Italian Population

Allele	Total (n = 201)
TT	0.38
tt	0.62

TABLE III

Distribution of the Three Genotypes in the Population

Genotype	Total number (%)	Normal number (%)	Osteoporotic number (%)
TT	22 (10.9)	9 (8.1)	13 (14.4)
Tt[a]	100 (49.7)	**64 (57.6)**[b]	36 (40)
Tt	79 (39.3)	38 (34.2)	**41 (45.5)**[c]
Total	201	111	90

Note. χ^2 Test = 6.5; p = 0.03.
[a] Tt was recognized as the major genotype in the total population.
[b] Tt genotype was the most frequent in normal women (bold).
[c] tt genotype was the most frequent in osteoporotic women (bold).

DISCUSSION

It is well known that genetic factors contribute to the achievement and maintenance of bone mass and that being osteoporosis a polygenic disorder more than one gene will have a role in determining peak bone mass and/or bone mass loss.

In the search for potential candidate genes a logical choice was the evaluation of genes encoding for calciotropic hormone receptors, bone matrix proteins and local regulators of bone metabolism. Indeed, several polymorphic genes, such as those encoding for VDR, ER,

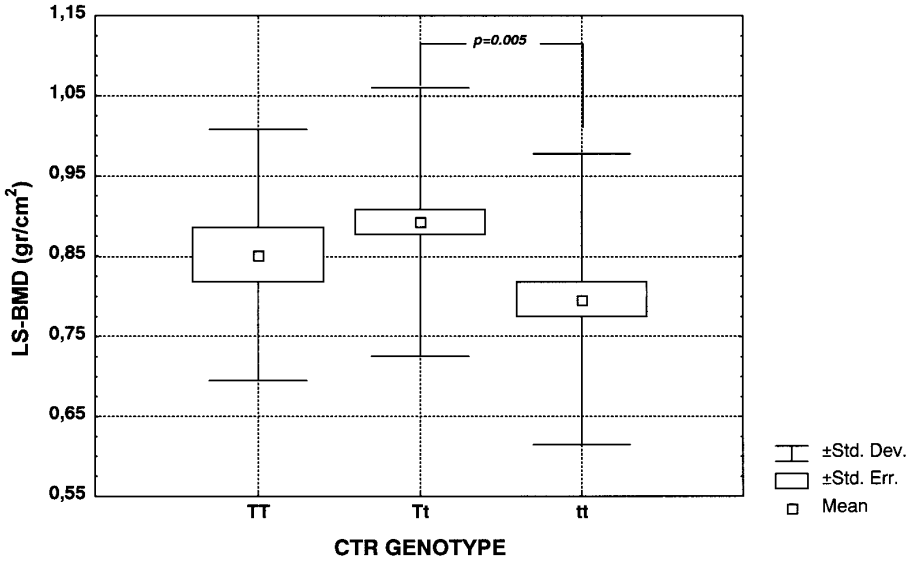

FIG. 2. Lumbar spine BMD values according to CTR genotypes. tt genotypes showed significantly lower lumbar-BMD in comparison with Tt (p=0.005) genotypes. The significance of difference between groups was calculates by Tukey's test.

collagen type I and IL6 have been linked to variation in BMD (1-9, 23-25). Studies on collagen type I have suggested that structural abnormalities in the genes controlling the structure and synthesis of procollagen type I, may be important in the pathophysiology of osteoporosis (26), as recently supported by the evidence of association between BMD and a polymorphic Sp1 site in the collagen type I gene (24). In agreement with the genetic relationship between the VDR gene locus and BMD (1-9), a significant association between fractional strontium absorption and VDR genotypes has recently been found (27). In addition, the association between BMD and polymorphisms of the ERα gene has been described (23) and more recently Mizunuma et al. (28) suggested that ERα gene is involved in accretion of BMD during young adulthood, but the effect is lost during menstrual transition. Finally, a variable number tandem repeat polymorphism in the 3′ flank of the IL-6 gene has been found to be associated with bone mass (25).

All these gene could play a positive or a negative role in modulating bone mass and in the development of osteoporosis. Allelic variants for a given gene could be analyzed alone or in combination with those of other genes recognized to modulate bone remodeling. In our experience the combined analysis of two genes encoding for steroid hormone receptors, the VDR and the ERα, made possible to recognize their interactions in BMD determination (9).

In the present study the gene encoding for a peptide hormone receptor, the CTR, has been evaluated for its potential role in BMD determination in a population of postmenopausal women. Recently, using SSCP analysis two alleles of human CTR gene have been identified in the French population but no association between the polymorphism recovered and development of osteoporosis was found (29). In addition Nakamura et al. (30) have described an Alu I RFLP at the CTR gene locus in the Japanese population. We recently observed significant differences on LS-BMD among various Alu I CTR allelic variants (31). In the present paper we described the presence of a previously unrecognized RFLP for the CTR gene by Taq I enzymatic digestion and Southern blotting using the cDNA probe encoding the full-length hormone CTR (12). This enzyme is able to detect a restriction site that we have indicated as T/t. This is characterized by a 7 kd (T) and a 5 kd (t) variant bands. In the present study, we found that genotype Tt was the most frequently represented in both osteoporotic and normal women. Women with tt genotype showed a 10.76% lower spinal BMD in comparison with those the Tt genotype. The magnitude of this effect was approximately of 0.096 gr/cm^2. No difference statistically significant were observed with TT genotype.

No significant variations of BMD among the genotypes were observed in the femoral neck BMD. This observation is in agreement with a stronger genetic influence on bone mass at sites with higher proportions of trabecular bone and with a genetic specificity for anatomical sites (32). In addition, several studies have demonstrated that after administration of calcitonin the most significant improvements in BMD values occurred at the spine with no effects at the appendicular skeleton (33). It is also likely that environmental factors (i.e. calcium intake and vitamin D metabolism) could play a more relevant role modifying femoral neck than lumbar BMD (29). Moreover, other quantitative

traits still not finely measurable, such as bone strength, bone quality and bone architecture appear to have a prominent role in determining fracture risk at appendicular sites (34).

The questions which follow these observations are: can the association of allelic variants of the human CTR and lumbar BMD be casual? Can, therefore, different CTR genotypes influence the response to the hormone? A constitutive activation of CTRs with enhanced production of cAMP and induction of a cAMP-responsive reporter gene in the absence of the agonist appear characterize the osteocalstic cells (34). Only few G-protein coupled receptor (GPCPs) have been identified with agonist indipendent activity form and these finding has led to a modification of the traditional receptor theory (36). It is now thought that receptors can exhibit at least two conformations, an inactive conformation (R), and an active conformation (R*) and that an equilibrium exists between these two states (29). It is, therefore, possible that constitutive activation of CTR, not dependent on the presence of CT in the environment, could be important in maintaining a basal CT activity. Allelic variability of CTR gene could modulate the activity of the constitutive CTRs influencing both the basal activity of the receptors and their response to the hormone. This would eventually lead to a lower BMD in tt subjects with reduced basal activity and/or decreased responsiveness to CT therapy.

Finally, it is known that CT increases 1-25 $(OH)_2$ D_3 production *in vivo* in humans (37) and that 24-hydroxylase activity is reduced after CT administration in rats (38). Conversely, the vitamin D status modulates the response of renal tubular cells to CT (39). The VDR and the CTR genes could, therefore, interact in modulating BMD like recently shown for the VDR and the ERα genes (9). Similarly we know that the CT reserve decreases progressively with age in both sexes (40) and that reduced estrogen levels at menopause can further contribute to decline of calcitonin secretion in females (41). In addition, estrogens are able to regulate calcitonin expression *in vivo* and ERs are expressed by C-cells but not by thyroid follicular cells (42). It is, therefore, possible that VDR and ER act in combination with the CTR gene in modulating bone mass.

The CTR gene becomes a new actor in the scenario of genes influencing BMD determination. As for other described genetic influences the history if just at the beginning and it is prudent to remain cautious about the significance of this correlation. These findings warrant further study of the Taq I CTR polymorphisms in larger populations and in other ethnic groups. Questions like causality and interactions with other genetic determinants need to be answered before such findings become useful in diagnosis of subjects at risk for osteoporosis and/or in the approach to the prevention and treatment of osteoporosis.

ACKNOWLEDGMENT

We are grateful to Dr. Anna Maria Morelli who generously supported our work with her knowledge and expertise.

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