Polymorphism of the Aromatase Gene in Postmenopausal Italian Women: Distribution and Correlation with Bone Mass and Fracture Risk*

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ABSTRACT
 Conversion of C19 steroids to estrogens is catalyzed by the aromatase enzyme. Inactivating mutations of the aromatase gene are associated with decreased bone mineral density in both men and women. Genetic studies suggest that several genes contribute to the regulation of bone mass via interaction with the modeling and remodeling processes. Among these genes, the aromatase gene is a potential candidate to be evaluated for segregation with bone metabolism and bone mass. A tetranucleotide simple tandem repeat polymorphism in intron 4 at the human aromatase cytochrome P-450 gene has been recently described. In the present study we evaluated the distribution of this polymorphism in a cohort of Italian postmenopausal women, both normal and osteoporotic. We observed that the NN genotype was significantly more frequent in nonosteoporotic women (72.7% vs. 27.2%), whereas the DN genotype was significantly more represented in osteoporotic women (90.48% vs. 9.5%; Pearson’s χ2 test = 42.8; df = 10; P < 0.01). The allele containing the longer TTTA repeats was statistically more represented in nonosteoporotic women (Pearson’s χ2 test = 19.14; df = 2; P = 0.00007). In addition, women with a high number of TTTA repeats had a significantly higher lumbar bone mineral density than women with alleles containing 8–11 TTTA repeats (P = 0.03). Finally, considering the spine fractures, a significantly higher incidence was observed in women with shorter TTTA repeats than in those with longer TTTA repeats (Pearson’s χ2 test = 7.3; df = 2; P = 0.02), equivalent to a relative risk of 4.1 (95% confidence interval, 1.19–13.87). In conclusion, the aromatase gene can be one of the several genes potentially involved in the maintenance of bone mass and in the regulation of bone mass loss. (J Clin Endocrinol Metab 86: 2263–2269, 2001)

ANDoRGENS AND estrogens are both important regulators of bone physiology (1). They account in part for sexual dimorphism of the skeleton influencing both growth and bone maintenance (1). Although estrogens and androgens are both believed to have direct effects on bone (2, 3), some androgens are aromatized to estrogens, raising the possibility that skeletal effects considered previously to be due to androgens may actually be due to estrogens. The enzyme complex aromatase comprises a specific form of cytochrome P450 and flavoprotein NADPH-reductase. It catalyzes the conversion of the Δ4-3-one A ring of androgens to the corresponding phenolic A ring typical of estrogens (4–6). Aromatase enzyme activity and its corresponding messenger ribonucleic acid (mRNA) have been shown in cultures of human osteoblast-like cells from adult and fetal bone, suggesting that estrogens are produced locally in bone (7–11). Glucocorticoids, 1α,25-dihydroxyvitamin D3 and chemokines, control aromatase activity and mRNA expression in bone (9, 12). In osteoblast-like cells and osteoclasts, the major promoter is found at exon 1.4 (5, 11). Other tissues use other promoters (13, 14).

Despite evidence to support a role for aromatase activity in bone cell metabolism, clinical examples of its importance are only now becoming available (15, 16). Inactivating mutations of the aromatase gene in both sexes are associated with increased bone turnover and decreased bone mineral density (BMD) (17, 18). Treatment of these patients with estrogen markedly improves bone mass (19–21). Moreover, aromatase expression in bone has been quantified with respect to osteoporosis as detected radiologically (22).

Genes involved in estrogen metabolism (the aromatase gene) and in estrogenic response (the estrogen receptor α gene) are possible contributors to the abnormal pathophysiological processes associated with osteoporosis (23, 24). Genetic variants in the human aromatase gene, for example, could alter estrogen metabolism. A tetranucleotide simple tandem repeat polymorphism in intron 4 of the human aromatase cytochrome P-450 gene has been recently described (25). The present study was designed to evaluate the distribution of this aromatase gene polymorphism in a cohort of normal and osteoporotic postmenopausal Italian women.

Materials and Methods

Three hundred and fifty postmenopausal women (mean ± SEM age, 57 ± 8 yr; range, 47–76 yr) were selected from 1700 women who were evaluated for osteoporotic risk that can be defined by radiological [x-ray and dual energy x-ray absorptiometry (DXA)] and biochemical exams to prevent and treat the disease. To adequately assess the role of aromatase in the genetics of osteoporosis and to minimize the influence of

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Bone densitometry and fracture assessment

Lumbar BMD (L2–L4) was measured by DXA (QDR 1000/W, Hologic, Inc., San Francisco, CA), with coefficients of variation of 0.5% in vivo and 0.9% in vitro. BMD at the upper femur (neck, Ward’s triangle, greater trochanter) was measured by DXA with coefficient of variation of 0.6% in vitro and 1.0% in vivo. A cross-calibration on the precision of measurements between the two centers in Florence and Siena was performed daily. The centers used personal spine phantom for calibration.

Vertebral fractures were evaluated by spine radiographs, according to the method of McCloskey (27). Fractures were present in both nonosteoporotic and osteoporotic women. Nonspine fractures were identified by self-report during the recruitment interview. Only nonviolent fractures were considered. The lateral lumbar spine x-ray was evaluated to detect osteophytes (28) and for facet joint osteoarthritis using a four-point scale (0 = none, 1 = mild, 2 = moderate, 3 = severe). Vascular calcifications were not evaluated because they have minimum impact on spinal density measurement (29).

Aromatase gene polymorphism

Genomic DNA was isolated from blood samples collected in ethylenediamine tetraacetate by a standard phenol-chloroform extraction procedure. PCR was performed using as primers GCAGGACTTAGCC-ACGCTTTCAG-3 and TTACAGTGAAGAGTCC5 (AAAT strand) (25). PCR amplification was carried out on 80 ng genomic DNA using 100 pmol of each oligonucleotide primer radiolabeled with [α-32P]dCTP using a random priming labeling kit (Roche, Mannheim, Germany).

Samples were processed as previously described (30), except that the denaturation cycle at 94°C was extended to 1.4 min. The PCR product was electrophoresed in a 6% polyacrylamide gel containing 7.6 mol/L of urea for 3 h at 30 watts. Genotypes were identified by autoradiography.

Statistical analysis

Aromatase TTTA repeat sequences were divided according to their distribution of aromatase genotypes in relation to the presence of peripheral and/or spine osteoporotic fractures did not show significant differences between genotypes in the 184 women analyzed (Pearson’s χ² test = 7.36; df = 5; P = 0.19). However, a trend characterized by a lower incidence of spine fractures was observed in NN genotype (data not shown).

Considering the number of the TTTA repeats patients were
grouped into categories as alleles containing TTTA repeats fewer than 8, between 8 and 11, and more than 11. Alleles containing the longer TTTA repeats were seen more frequently in nonosteoporotic women compared with osteoporotic patients (Pearson’s $\chi^2$ test: 0.32; df = 2; $P = 0.08$), even though the incidence of fractures in women with alleles containing the longer TTTA repeats was lower. Moreover, considering only spine fractures, a significantly higher incidence was observed in the women with shorter TTTA repeats (spine fractures: $+ = 38$; $- = 3$) in comparison with longer TTTA repeats (spine fractures: $+ = 11$; $- = 22$) (Pearson’s $\chi^2$ test: 7.3; df = 2; $P = 0.02$), equivalent to a relative risk of 4.1 (95% confidence interval, 1.19–13.87; Fig. 3).

Applying ANCOVA, significant differences among the genotypes were observed in mean BMD at the lumbar spine ($P = 0.001$), but not at the femoral neck site ($P = 0.74$). Tukey’s test used to compare the six genotypes after ANCOVA analysis showed that women with the DN genotype had a significantly lower BMD in comparison with NN ($P = 0.02$) and CN ($P = 0.008$) genotypes (Fig. 4). No statistically significant differences were observed between the genotypes at the femoral neck BMD. The same test used to compare the groups with different numbers of TTTA repeats showed that women with a high number of TTTA repeats had a significantly higher lumbar BMD than women with alleles containing 8–11 TTTA repeats ($P = 0.03$; Fig. 5).

ANCOVA was also applied to evaluate whether the role of aromatase genotypes in lumbar BMD was influenced by YSM. On the basis of YSM, women were divided into 3 groups: less than 5, between 5 and 10, and more than 10 YSM. A statistically significant segregation of aromatase genotypes and lumbar BMD was observed only in patients in the first 5 YSM ($P = 0.02$), with women with DN genotype showing a significantly lower BMD than those with the NN genotype ($P = 0.017$; Fig. 6).

**Discussion**

Bone remodeling is regulated by systemic hormones and locally produced factors acting in concert to maintain bone mass. Among hormones, estrogens exhibit recognized major effects on bone metabolism, not only in women but also in men (31). Postmenopausal women with undetectable serum estradiol concentrations have a high risk of developing hip and vertebral fractures (32, 33). Indeed, a male, a patient with a homozygous estrogen receptor $\alpha$-inactivating mutation was reported to have a marked decrease in his BMD (34).
Similarly, inactivating mutations of the aromatase gene were associated with low BMD in males as well as in females (17, 19). In fertile women the ovary represents the major source of circulating estrogens, whereas in postmenopausal women extraglandular aromatization of circulating androgens becomes the most important metabolic mechanism for estrogen production (35). Bone tissue and bone-derived cells express aromatase gene and enzyme activity (9, 11). It is, therefore, likely that estrogen production in bone tissue could result in local regulation of bone remodeling during life (9). With these conditions, the gene encoding aromatase becomes a potential candidate to be evaluated in the attempt to elucidate the genetic background of osteoporosis. In the present study the distribution of a tetranucleotide repeat polymorphism of the human aromatase gene was evaluated in a population of Italian postmenopausal women. The nonosteoporotic and osteoporotic populations were homogeneous for characteristics such as weight, height, age, YSM, and ethnicity. Of the six major allelic variants, the N allele was shown to be most prevalent in nonosteoporotic women, suggesting its independent protective function for susceptibility to osteoporosis. A mechanism through which the N allele acts as a protective factor could be the capability of higher local estrogen synthesis in bone tissue of subject bearing N allele(s). Indeed, the homozygous NN genotype was significantly more frequent in nonosteoporotic women than in osteoporotic women. In agreement with this finding, alleles containing longer (>11) TTTA repeats (mostly represented by the N allele) were more prevalent in nonosteoporotic women.

ANOVA to evaluate lumbar and femoral neck BMD differences among the six major genotypes of the aromatase gene showed a significant difference in genotype distribution at the lumbar spine. There was no correlation with femoral neck BMD. ANCOVA confirmed these results. Applying Tukey’s test to compare the six genotypes after ANCOVA analysis, we observed that women with the DN genotype showed significantly lower lumbar BMD than those with NN and CN genotypes. In agreement with these results is the fact that women with a high number of TTTA repeats had a higher lumbar BMD than women with allele containing TTTA repeats between 8 and 11. In particular, lumbar spine BMD was approximately 0.061 g/cm² (7%) higher in women with a high number of TTTA repeats than in women with alleles containing between 8 and 11 TTTA repeats.

BMD at the lumbar spine was approximately 0.193 g/cm²
(21%) lower in DN individuals than in those with NN and 0.140 g/cm² (16.1%) lower than in those with CN subjects. A difference of this magnitude could increase long-term fracture risk in DN women compared with NN and CN patients. In the present study we evaluated potential differences between genotypes and the incidence of osteoporotic fractures. A total of 184 women affected by fractures selected from both osteoporotic and nonosteoporotic groups were analyzed. We did not observe significant differences among genotypes for the incidence of any site osteoporotic fractures (Pearson’s χ² test = 0.32; df = 2; P = 0.08). However, the allele with high number of TTTA repeats had a significantly lower incidence of spine fractures (spine fractures: + = 38; − = 3; Pearson’s χ² test = 7.3; df = 2; P = 0.02).

Our inability to detect a difference is most likely due to the small size of the sample analyzed. The role of genotype NN in protecting women from postmenopausal bone loss and consequently from risk of developing vertebral fractures is still unknown.

No statistically significant differences in bone density were observed among the six genotypes at the femoral neck. Similarly, women with different numbers of TTTA repeats had femoral neck BMD not statistically different from each other. These results are in keeping with other observations (36). In view of the most rapid loss of lumbar spine bone density due to estrogen deficiency, the aromatase gene could be involved in early postmenopausal bone loss rather than that in the later postmenopausal years. This hypothesis was
confirmed by statistical analysis showing a significant segregation of the aromatase genotypes and lumbar BMD only in the first 5 YSM, with the DN genotype showing a lumbar BMD lower by 0.169 g/cm² (16.7%) compared with the NN genotype.

The mechanism through which aromatase gene activity associated with this polymorphic site controls bone metabolism is obscure. Theoretically women with DN genotype could be characterized by lower aromatase activity and/or synthesis with consequent reduction of estrogen synthesis. The aromatase gene could, therefore, be pivotal in the maintenance of a sufficient amount of local estrogens in bone tissue. Interestingly, the allelic variant containing longer TTTA repeats segregates with breast cancer risk (37, 38). It is likely that patients with allele containing longer TTTA repeats could express higher aromatase activity with increased estrogen production, which should be protective for bone loss while increasing the risk of breast cancer. This interpretation is consistent with the relationship between breast cancer risk and higher BMD (39). In contrast with this interpretation is the lack of correlation between estradiol concentrations and osteoporosis in postmenopausal women (9). Possibly, local bone tissue estrogens more than circulating concentrations may be important in the regulation of bone turnover in postmenopausal women, as estrogens produced locally could act as autocrine or paracrine modulators of bone cells. Functional studies in the future will have to encompass analysis of differential aromatase expression, activity, and regulatory pathways in the presence of different gene polymorphisms.

Finally, several studies are now showing that the age-related decline in male BMD is mostly related to declining estrogens levels (40, 41). It is likely that the effects of estrogens in the male might be due to a balance between local and peripheral estrogen production, and polymorphisms of the aromatase gene in women might have significance for men. There is clear evidence of genetic modulation of bone phenotype parameters, including bone density, quantitative ultrasound, bone size, and bone turnover at any particular age phase of life; genetic factors explain about 70% of the variance in bone phenotype. The importance of genetic heterogeneity, including ethnicity, as well as environmental and confounder factors need to be taken into account in gene search approaches (42).

It is well known that multifactorial diseases such as osteoporosis involve multiple genes and environmental factors and result principally from genetic variations that are relatively common in the general population. Linkage analysis and association studies are the two analytical methods available to detect the specific genetic regions, and candidate genes responsible for complex diseases present several crucial differences. In fact, association studies test whether a disease and an allele show correlated occurrence in a population, whereas linkage studies test whether they show correlated transmission within a pedigree (43). Association analysis for complex diseases may be an efficient approach to recognize genes with major impact even though association studies show some limitations (44), such as ethnical homogeneity and sample size. All of these arguments were considered, and these limitations were carefully evaluated in the analysis performed in the present study. In addition, as suggested by some researchers (43), to prevent spurious associations arising from admixture and given the difficulty of selecting a control group that is perfectly matched for ethnic ancestry, we use as an internal control for allele frequency evaluation the analysis of both affected individuals and their
parents. Together, these considerations make it possible to ascribe the aromatase gene to the number of genes involved in the control of postmenopausal bone loss. Association studies are most meaningful when applied to functionally significant variations in genes having a clear biological relation to the trait (43). For this reason, functional studies to evaluate the activity and the expression of aromatase mRNA in fibroblasts of patients with different genotypes are underway in our laboratory. Undoubtedly, the genetic basis of osteoporosis will be found to be associated with a panoply of genes, all of which contribute to the genetic and phenotypic aspects of the postmenopausal women.

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