CLINICAL STUDY

A common polymorphism in the 5'-untranslated region of the aromatase gene influences bone mass and fracture risk

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Abstract

Objective: The aromatization of androgenic precursors in peripheral tissues, including bone, is the main source of estrogens after the menopause. CYP19, the gene encoding aromatase, has a long 5'-untranslated region with several variants of exon I and specific promoters. The aim of this study was to investigate the possible relationship between a common biallelic (C/G) polymorphism located on exon I.2 and bone mineral density (BMD).

Design: This was designed to be an association study between CYP19 polymorphism and BMD and the risk of vertebral fractures in women.

Methods: DNA was extracted from the peripheral blood of 299 women (116 premenopausal and 183 postmenopausal). CYP19 alleles were identified by a method based on the exonuclease activity of Taq-polymerase. BMD was determined by dual-energy absorptiometry.

Results: In premenopausal women there were no genotype-related differences in BMD. However, postmenopausal women with the CC genotype had lower spine and hip BMD than those with the GG genotype. The association between CYP19 genotypes and BMD was independent of other variables, such as age, height, body weight, calcium intake or years since menopause. The CC genotype was also associated with an increased risk of osteoporotic vertebral fractures (odds ratio 2.0; $P = 0.03$). Serum levels of estrone and estradiol were similar in women with CC and GG alleles.

Conclusions: A common biallelic polymorphism in the 5'-untranslated region of the CYP19-aromatase gene was associated with significant differences in bone mass and the risk of vertebral fractures in postmenopausal women. Given the frequency of allelic variants, genotype-related differences appear to be important from the perspective of the individual as well as the general population. Further studies are needed to elucidate underlying mechanisms that may be dependent on differences in estrogen bioactivity at the bone tissue level.

European Journal of Endocrinology 150 699–704

Introduction

In humans, bone mass increases during the growth period, reaching a peak by the third decade of life, and then decreases with aging. Estrogens inhibit bone resorption and exert an important influence on bone homeostasis. Thus, women experience a rapid bone loss after the decrease in ovarian estrogen production following natural or surgical menopause (1).

The ovary is the main source of estrogen in premenopausal women. However, after the menopause, synthesis in peripheral tissues becomes more important to maintain estrogen levels, although they are lower than those attained before the menopause. In fact, adipose and other tissues express aromatase, the product of the CYP19 gene, a critical enzyme in the peripheral synthesis of estrogens. It is responsible

for catalysing the aromatization of C19 androgens to C18 estrogens such as estradiol and estrone (2, 3). Estrone is the most abundant estrogen in the serum of postmenopausal women. It is formed by the aromatization of androstenedione and other precursors released by the adrenal glands and, in smaller amounts, by the ovaries (4).

Although overall estrogen bioactivity markedly decreases after the menopause, estrogens appear to be important in bone homeostasis in postmenopausal women. In some cross-sectional studies, a relationship has been found between serum estrogens and bone mass (5). The role of aromatase has also been highlighted by experimental and clinical studies. Knock-out ARKO mice, which do not express aromatase, develop osteopenia (6, 7). Also, women with breast cancer treated with aromatase inhibitors seem to have

an increased bone turnover, and suffer a loss of bone mass and an increased incidence of fractures (8, 9).

CYP19 has a complex structure, with a long 5'-untranslated region that serves as the regulatory unit of the gene (2, 10, 11). It contains different variants of the first exon. There are tissue differences regarding the alternative use of multiple promoters which regulate mature aromatase mRNA expression by splicing each first exon variant onto a common splice junction situated in exon II, immediately upstream of the coding region. Thus, although the aromatase protein seems to have the same sequence in all cases, the levels of protein synthesis vary, depending on the tissue studied and the presence of paracrine and hormonal factors modulating RNA expression. It has been recently demonstrated that some bone regulatory factors, including vitamin D metabolites and glucocorticoids, modulate the expression of CYP19 in bone cells. Those data prompted us to explore the possibility that common polymorphisms in the CYP19 gene could be associated with bone mass.

Materials and methods

Subjects

The study group included 299 women. Of them, 116 were healthy premenopausal women (aged 22–45 years) and 183 postmenopausal women (aged 61–86 years), recruited from volunteers and patients with primary postmenopausal osteoporosis ($n = 107$), including 65 with osteoporotic vertebral fractures. Patients with a present or past history of diseases known to affect skeletal homeostasis, or who were taking drugs able to interfere with bone metabolism (for example, glucocorticoids, anti-epileptics, estrogens or thiazides) were excluded. Physical activity and calcium intake from dairy products were estimated using questionnaires. Bone mineral density (BMD) was estimated by X-ray absorptiometry (Hologic, Waltham, MA, USA) at the lumbar spine (L2–L4) and the hip. Hip T-scores, i.e. BMD expressed as the number of standard deviations below the peak BMD of young women, were estimated using the values of Caucasian women in the third National Health and Nutrition Examination Survey (NHANES) study as reference (12). T-scores at the lumbar spine were estimated using a Spanish population database as reference (13). Verbal informed consent was obtained from participants. The study protocol was approved by the Institutional Research Committee.

DNA analysis

We were interested in common variations that could be important as risk factors for osteoporosis from a population perspective (i.e. present in at least 15% individuals). We therefore searched public databases looking

for polymorphisms in the 5'-untranslated region of the CYP19 gene with a frequency of the least prevalent allele of at least 40%. A biallelic C/G polymorphism (National Center for Biotechnology Information database, rs1062033) was chosen as the study target. DNA was extracted from peripheral blood by the Qiagen method (Hilden, Germany), and alleles were typed by a procedure based on the exonuclease activity of Taq-polymerase, using allele-specific Taq-Man probes labeled with VIC and FAM (Applied Biosystems, Warrington, Cheshire, UK). Amplification reactions were performed in a 5 μ l volume, following the manufacturer's instructions, but conditions were slightly modified (annealing time was increased up to 30 s and the number of PCR cycles to 48). After amplification, alleles were typed by reading the fluorescence on an ABI 7000 sequence detector (Applied Biosystems). In about 7% of cases, the results were ambiguous and samples had to be re-typed. Random samples were analysed twice to check for consistency of results.

Serum estrogens

Estradiol levels were measured by RIA (Diagnostic Systems Laboratories, Webster, TX, USA), with a detection limit of about 1 pg/ml and 4–9% coefficient of variation. Serum estrone levels were measured by a sandwich ELISA (IBL, Hamburg, Germany), which has a sensitivity of 15 pmol/l. Interassay coefficient of variation is 10%.

Statistics

ANOVA was used to test for differences in BMD among subjects with different genotypes, followed by a test for linear trend. The results were adjusted for potential confounding variables by analysis of covariance (ANCOVA) and multivariate regression analysis. χ^2 test was used to test differences among proportions. Odds ratios (OR) were estimated by logistic regression analysis. All tests were performed with SPSS software (SPSS Inc., Chicago, IL, USA).

Results

Frequencies of the C and G alleles, estimated by the counting method, were 57.8% and 42.2% respectively. The overall frequencies for CC, CG and GG genotypes were 33.9%, 47.8% and 18.3% respectively. There was no evidence of departure from the Hardy–Weinberg equilibrium.

In premenopausal women, no differences in either hip or spine BMD were found among subgroups with different genotypes (Table 1 and Fig. 1).

However, among postmenopausal women there were marked CYP19-related differences in BMD at both regions. Allele C was associated with a lower bone

Table 1 Weight, height and BMD at the lumbar spine and total hip regions in premenopausal women, according to CYP19 genotype. Values are means with s.d. in parentheses.

	CC	CG	GG
Number	38	53	25
Age (years)	33 (7)	35 (8)	36 (7)
Weight (kg)	61 (8)	59 (10)	59 (7)
Height (cm)	163 (8)	162 (7)	160 (5)
Spine BMD (g/cm ²)	1.045 (0.118)	1.035 (0.117)	1.022 (0.116)
Hip BMD (g/cm ²)	0.936 (0.100)	0.918 (0.108)	0.925 (0.106)

No significant differences.

Figure 1 BMD at the spine and different hip regions in premenopausal (top) and postmenopausal women (bottom). Values are means and S.E.M. of T-scores. Troch, trochanter region; inter, intertrochanteric region; ns, not significant.

mass. Thus, the group with the CC genotype had the lowest BMD, whereas those with the GG genotype had the highest BMD and intermediate values were found among those with the CG genotype. Similar results

were found at the different hip subregions studied (Table 2 and Fig. 1). There were no differences in age, duration of menopause, exercise level, calcium intake or anthropometric variables (Table 2). Body weight, age and the number of years since menopause were also associated with hip and spine BMD. Nevertheless, the association between CYP19 genotypes and BMD remained highly significant after adjustments by those factors were made. The CYP19 genotype explained 7.5% and 5.3% of the variance at the spine and the hip respectively (Table 3). The CC genotype was associated with an increased risk for osteoporosis, both at the lumbar spine (adjusted OR 3.4, 95% confidence interval (CI) 1.5–7.5, $P = 0.003$) and the hip (adjusted OR 4.0, 95% CI 1.3–12.6, $P = 0.02$).

No interaction between body weight and CYP19 genotypes was detected. When women were divided into subgroups according to body mass index, the same trend towards an association between genotypes and BMD was present in all subgroups, although it did not always reach the conventional level for statistical significance, probably due to the smaller size of the subgroups (Fig. 2).

The analysis of genotype distribution among the subgroups of postmenopausal women revealed that the CC genotype was not only associated with a lower BMD but it was also over-represented in the subgroup of women with osteoporotic vertebral fractures. The CC genotype was present in 46% of postmenopausal women with fractures, but only in 30% of postmenopausal controls (OR 2.0; 95% CI 1.1–3.8, $P = 0.03$) (Table 4).

Serum estrogen levels were measured in a subset of 46 homozygotic postmenopausal women. There were no differences in serum estradiol between women with CC and GG genotypes (27 ± 7 vs 27 ± 8 pg/ml), or in serum estrone levels (92 ± 33 vs 90 ± 32 pg/ml).

Discussion

The role of aromatase-dependent synthesis of estrogens has been clearly revealed by the phenotype

Table 2 Characteristics of postmenopausal women with different CYP19 genotypes. Values are means with s.d. in parentheses.

	CC	CG	GG	P
Number	61	92	30	
Age (years)	72 (7)	72 (7)	72 (7)	ns
Years since menopause	22 (8)	20 (7)	22 (7)	ns
Height (cm)	153 (6)	153 (7)	154 (5)	ns
Weight (kg)	67 (11)	66 (10)	66 (10)	ns
Calcium intake (mg)	828 (422)	680 (373)	769 (438)	ns
Spine BMD (g/cm ²)	0.758 (0.130)	0.795 (0.154)	0.848 (0.194)	0.01
Neck (g/cm ²)	0.626 (0.108)	0.644 (0.099)	0.671 (0.111)	0.05
Trochanter (g/cm ²)	0.539 (0.126)	0.573 (0.107)	0.600 (0.124)	0.01
Intertrochanter (g/cm ²)	0.871 (0.183)	0.921 (0.156)	0.956 (0.173)	0.02
Total hip (g/cm ²)	0.735 (0.149)	0.776 (0.126)	0.814 (0.147)	0.01

ns, not significant.

Table 3 Independent predictors of BMD in postmenopausal women. CYP 19 genotypes were categorized with numbers (CC: 1, CG: 2, GG: 3). The standardized coefficients of regression (β), *P* values and partial coefficients of regression (*r*) are shown.

	Lumbar spine			Total hip		
	β	<i>P</i>	<i>r</i>	β	<i>P</i>	<i>r</i>
Weight	0.46	<0.001	0.47	0.47	<0.001	0.49
Years since menopause	0.13	0.07	0.15	-0.14	0.04	-0.18
CYP19 genotype	0.25	0.001	0.27	0.20	0.005	0.23

Figure 2 Spine BMD of postmenopausal women according to body mass index (BMI) and CYP19 genotype.

abnormalities described in cases of rare null mutations (for review see 14). However, although it is important to improve the knowledge of physiological mechanisms, those cases are extremely infrequent and have little relevance from a population perspective. Gene variations more frequent in the population, albeit associated with smaller phenotypic differences, may be more relevant when studying polygenic diseases, such as osteoporosis. Our results indicate that rs1062033 alleles are associated with clinically relevant differences in the BMD of postmenopausal women. However, no differences were found among young women. Such an age-dependent influence is consistent with the current knowledge about the biology of sex steroids. In young individuals, the gonads synthesize and release to the circulation large amounts of estradiol which act on target tissues, including bone. Ovarian-derived estrogens appear to be critical in determining peak bone mass. Indeed, when hypogonadism develops prior to

Table 4 Association between CYP19 genotypes and osteoporotic vertebral fractures (number with percentage of postmenopausal women in parentheses).

	CC	CG	GG
With fractures	30 (46)	27 (42)	8 (12)
Without fractures	35 (30)	62 (52)	21 (18)

puberty or during adolescence, a subnormal bone accretion results (1).

However, after the menopause the peripheral synthesis of estrogens acquires greater relevance. Gonadal synthesis of sex steroids declines and most estrogen originates from the peripheral aromatization of less abundant adrenal androgenic precursors. Furthermore, aromatase expression in adipose tissue increases with aging (15) and this is frequently considered as the main source of circulating estrogen in old individuals. Nevertheless, it must be emphasized that other cells, including bone cells, express aromatase and other enzymes required for estrogen synthesis. Indeed, aromatase activity in cultured osteoblasts is similar to that present in adipose stromal cells (16). Sex steroids therefore appear to have two types of effect: some of them can be considered as 'hormonal', related to circulating levels; others are local effects, autocrine, paracrine or even intracrine, related to the synthesis in target tissues, but not necessarily related to estrogen levels in the circulation (17). In the present study we have not found any differences in the circulating estradiol or estrone levels among women with different genotypes. Thus, marked differences in BMD between women with different CYP19 genotypes are likely related to differences in estrogen bioactivity at the bone tissue level.

The CYP19 gene is located in chromosome 15q21.2 and comprises ten exons (11, 17). The common initiation of the translation site is located in exon II. There are at least nine variants of exon I. Transcription in the gonads is driven by a proximal promoter and transcripts contain, at the 5'-end, a sequence that is immediately upstream of the initiation of the translation site. However, in other tissues transcription is driven by distal promoters, located up to 90 kb upstream in the 5'-untranslated region. Thus, upstream of exon II there are a number of alternative first exons that can be spliced into the 5'-untranslated region in a tissue-specific fashion. Exon I.4 is the preferential transcript in adipose tissue, which also transcribes PII, I.3 and I.6 in smaller amounts (3, 10, 18). The main promoter used in bone tissue seems to be I.4, but bone cells have also been shown to express exons PII, I.6, I.3, I.2 and I.f in some culture conditions (16, 19, 20). In fact, a phenomenon of promoter switching depending upon the regulatory factors present in the media, including hormones and cytokines, and cell differentiation inducers has been shown in many tissues.

The polymorphism studied here was located 13 kb upstream of exon 2, and maps to exon I.2 (Genbank sequence S96437), 295 bp from the GTAAG of the donor splice sequence and 85 bp downstream of a putative TATA box (21). Expression of exon I.2 was first reported in the placenta, but it is also expressed in other tissues, including bone. Indeed, Enjuanes *et al.* (20) have recently reported that 1,25-dihydroxyvitamin D3 and glucocorticoids induce I.2 expression by

normal human osteoblasts. Moreover, Shozu *et al.* (22) reported that the human leukemic THP-1 cell line, which can be induced to differentiate towards an osteoclastic phenotype by vitamin D, expresses I.2 and other Cyp19 exon I untranslated transcripts. Some of the I.2 transcripts were indeed mixed transcripts containing 63–370 bp of the 3'-end of exon I.4 sequence upstream of untranslated exon I.2, a phenomenon also observed in adipose tissue.

The mechanism underlying the association between CYP19 alleles and bone mass is unclear. It could be directly related to differences in the transcription response to promoters. Also, given the results by Enjuanes *et al.* (20), it is tempting to speculate that it could be due to differences in the response of bone cells to calciotropic hormones, but this remains merely speculative. Alternatively, the effect could be mediated by the linkage of this SNP with other regulatory regions of the gene. It is interesting to note that, in a preliminary analysis of a subset of women, we found a disequilibrium linkage between the SNP in exon I.2 and a tetranucleotide repeat in intron 4 of CYP gene (not shown). Albeit without known functional relevance, the polymorphism in intron 4 was previously associated with osteoporosis risk in Italian women (23). Although the result has not been confirmed in another recent study in Finland (24), it is possible to speculate that the linkage with rs1062033 or other polymorphisms in the regulatory untranslated regions of the CYP19 gene mediates the association between the intronic microsatellite and bone mass.

In conclusion, a common biallelic polymorphism in the 5'-untranslated region of the CYP19 gene is associated with significant differences in bone mass and the risk of vertebral fractures in postmenopausal women. No differences existed in young women, indicating that the polymorphism is associated with postmenopausal bone loss rather than to the peak bone mass attained in early adulthood. Given the frequency of allelic variants, genotype-related differences appear to be important from the perspective of the individual as well as the general population. Further studies are needed to elucidate the underlying mechanisms.

Acknowledgements

This study was supported by a grant from the Fondo de Investigaciones Sanitarias (PI 020063). We thank Carolina Sañudo and Blanca Paule for technical assistance.

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Received 15 October 2003

Accepted 2 February 2004