Association of *CYP19* Gene Polymorphism with Vertebral Fractures in Japanese Postmenopausal Women

**Yasuko Koudu · Tsuneko Onouchi ·
Takayuki Hosoi · Toshiyuki Horiuchi**

Abstract This study investigates aromatase gene polymorphism, which might influence bone strength in terms of mineral density and quality. We explored the relationship between *CYP19* polymorphisms and vertebral fractures in postmenopausal Japanese women. In addition, we compared estrogen and testosterone levels in Japanese postmenopausal women with and without fractures. Osteoporotic postmenopausal women showed higher incidences of vertebral fractures than osteopenic women or women with normal lumbar bone mineral density (L2-4 BMD). Estrogen concentrations in postmenopausal women were associated with BMD; however, no association was found between sex hormone levels and the presence of fractures. The C allele rs2470152 was significantly associated with increased risk of vertebral fractures ($P = 0.04$), whereas none of the *CYP19* polymorphisms showed differences in sex steroid levels between subjects with and without fractures. Allelic variants of aromatase genes appear to interact to influence the risk of vertebral fractures in postmenopausal Japanese women.

Keywords Aromatase gene polymorphisms · Vertebral fractures · Postmenopausal women

Y. Koudu · T. Horiuchi (✉)
Department of Endocrinology and Metabolism, Tokyo Metropolitan Toshima Hospital,
33-1 Sakaecho Itabashiku, Tokyo 173-0015, Japan
e-mail: t-horiuchi@toshima-hp.jp

T. Onouchi
Department of Collaborative Research, Teikyo University School of Medicine, Tokyo, Japan

T. Hosoi
Department of Advanced Medicine, National Center of Geriatrics and Gerontology, Obu, Japan

Introduction

Osteoporosis is caused by multiple factors, including environmental factors (such as calcium intake), exercise, and estrogen levels. The main source of estrogen in postmenopausal women is the aromatization of androgenic precursors, a reaction catalyzed by the cytochrome P450 (CYP) aromatase enzyme, encoded by *CYP19* located on chromosome 15q21.1. It has recently been reported that estrogen levels are genetically determined by aromatase activity (Olson et al. 2007; Haiman et al. 2007; Sowers et al. 2006). In addition, allelic variants of the aromatase gene have been associated with bone mineral density (BMD) and bone fractures (Hong et al. 2007; Masi et al. 2001; Somner et al. 2004). A/G polymorphisms in the 3′ untranslated region (UTR) and the I.2 promoter (rs10046 and rs1062033; Rinancho et al. 2005) and an A/G polymorphism in the I.6 promoter rs4775936 (Enjuanes et al. 2006) of the aromatase gene have been studied in relation to osteoporosis and BMD, but the results remain controversial. In addition, an rs2470152 polymorphism in the aromatase gene has been shown to affect serum estrogen levels in Swedish men (Eriksson et al. 2009). Therefore, in order to analyze the association with the risk of vertebral fractures in postmenopausal women, we conducted a cross-sectional study of the interaction between *CYP19* gene polymorphisms and sex steroid hormone levels or risk of vertebral fractures in Japanese postmenopausal women. In this study, we focused on four markers (rs2470152, rs4775936, rs1062033, and rs10046) to clarify the association between polymorphisms in aromatase genes and vertebral fractures.

Materials and Methods

Study Subjects

Three hundred sets of genomic DNA and serum samples were provided from the collected samples of the Institute of Medical Sciences, Tokyo University, obtained for tailor-made medicine realization projects. These samples were collected from the various institutions that were members of these projects following the approval of the individual ethics committees. Ethical approval was obtained from the Ethics Committee of the Leading Project for Personalized Medicine in the Institute of Medical Science, University of Tokyo, and the Tokyo Metropolitan Geriatric Hospital. Another 300 DNA samples were collected from women for the purpose of analyzing the relationship between polymorphisms and the etiology of disease in the Japanese population. The samples were provided by the Leading Project for Personalized Medicine of the Ministry of Education, Culture, Sports, Science and Technology, Japan.

The samples were divided into three categories according to the *T* score of the measurement of lumbar spine BMD (L2-4 BMD) by dual energy X-ray absorptiometry (DXA) as defined by the World Health Organization: *T* scores of −1.0 and above were classified as normal BMD, scores of −2.5 to −1.0 were considered osteopenia, and scores below −2.5 were considered osteoporotic.

For assessment of vertebral fractures, anteroposterior and lateral X-ray examinations of the thoracic and lumbar spine were performed. Morphometrically,

a vertebral fracture was defined in terms of the ratio of the anterior height of vertebral body to the posterior height (below 0.75) or the ratio of the center height to the anterior or posterior height (below 0.8). In all cases, the vertebral fractures were evaluated by two groups of radiologists and geriatricians in each institute.

Sex Steroid Assay

The serum levels of testosterone (T) and estradiol (E_2) were measured by mass spectrophotometry (LC–MS/MS). Bioavailable testosterone and estrogen, which includes the free form and the albumin-binding form, were measured by LC–MS/MS (Arai et al. 2010). Serum samples were stored at $-70°C$ until analyzed. For statistical analysis, the values were transformed into logarithmic form, since the values are exponential and the distributions of T and E_2 levels were skewed using the raw data.

Genotype Analysis

We examined four polymorphisms of *CYP19*: rs1062033, a G/C SNP located at around exon 1.2 (at position chromosome 15, 49335230); rs10046, a T/C SNP located in the 3′ UTR (at position chr.15, 49290276); rs4775936, a C/T SNP located in the vicinity of exon 1.6 (at position chr.15, 49323314); and rs2470152, a T/C SNP located in intron 1 (at position chr.15, 49382254). These SNPs were identified by searching the National Center for Biotechnology Information (NCBI) database because they are analyzable by the readily available TaqMan assays used for disease association studies (Applied Biosystems). Polymorphisms in genomic DNA were measured by the TaqMan assay. Age, body mass index, and years since menopause were examined in three SNP genotypes among four *CYP19* markers.

Statistical Analyses

Chi-square analysis was used to compare the numbers of osteoporosis, osteopenia, and normal patients by T scores of L2-4 BMD with and without fractures. Similarly, each parameter was compared among the three genotypes in four *CYP19* markers using ANOVA. The correlation between estradiol levels and L2-4 BMD was shown using Pearson's coefficients. The associations between aromatase gene polymorphisms and vertebral fracture risk were compared by Chi-square analysis using SPSS software.

Results

Bone Density Data

There were significantly more women with fractures than without among patients with osteoporosis ($T < -2.5$; $P < 0.05$), and there was no significant increase in fractures among normal patients or those with osteopenia. There were no differences

in the log estradiol (Log E_2) or log testosterone (Log T) values between women with fractures and those without fractures (Table 1).

Relationship Between L2-4 BMD and Estrogen level

Log E_2 levels in postmenopausal women were significantly associated with L2-4 BMD ($r = 0.21$, $p = 0.03$; Fig. 1), whereas log T levels showed no association (data not shown).

Genotype Analysis

When we examined the correlation between the four polymorphisms (rs2470152, rs1062033, rs4775963, and rs10046) and vertebral fractures in postmenopausal women, we found a significant correlation for rs2470152 ($P = 0.04$) but not for the

Table 1 Bone mineral density of postmenopausal women with and without fractures

[a] Bone mineral density was measured in 89 of the 275 subjects

NS not significant

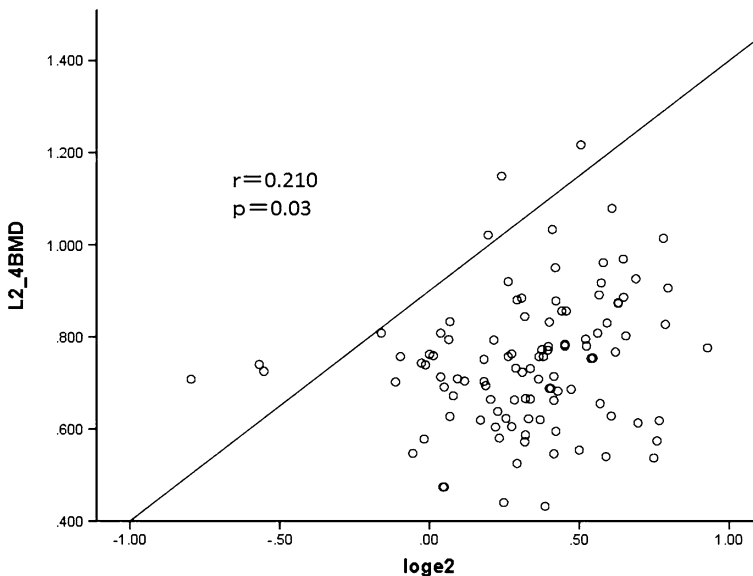| T score[a] & sex steroids	Women without fractures (137)	Women with fractures (138)	P
$T < -2.5$	18	37	0.015
$-2.5 \leq T < 1.0$	12	13	NS
$-1.0 \leq T$	4	5	NS
Log E_2 (pg/ml)	0.335 ± 0.383	0.327 ± 0.330	NS
Log T (ng/dl)	2.033 ± 0.367	2.067 ± 0.247	NS

Fig. 1 Correlation between log E_2 and L2-4 BMD in postmenopausal women. Estrogen levels were significantly correlated with L2-4 BMD ($r = 0.21$, $p = 0.03$)

Table 2 Correlation of four *CYP19* SNPs with vertebral fractures

SNP	Genotype	Premenopause		*P*	Postmenopause		*P*
		Control (*n* = 19)	Case (*n* = 6)		Control (*n* = 136)	Case (*n* = 138)	
rs2470152	CC	9	1	0.408	44	47	0.040[a]
	TC	8	4		56	71	
	TT	2	1		36	20	
rs4775936	CC	5	1	0.712	48	59	0.416
	CT	9	4		68	59	
	TT	5	1		20	20	
rs1062033	CC	4	1	0.693	47	56	0.507
	CG	9	4		64	62	
	GG	6	1		25	20	
rs10046	TT	7	1	0.550	31	26	0.324
	TC	8	4		67	62	
	CC	4	1		38	50	

[a] Only rs2740152 polymorphisms of the aromatase gene showed a significant correlation with vertebral fractures ($P = 0.04$)

Table 3 Characteristics of postmenopausal Japanese women and three SNPs of rs2470152

Characteristic	Genotype			*P*
	CC	CT	TT	
Age (years)	72.8 ± 9.3	73.6 ± 8.3	74.3 ± 6.8	NS
Body mass index (kg/m^2)	22.4 ± 4.6	21.8 ± 4.5	21.1 ± 4.3	NS
Years since menopause	24.2 ± 9.5	24.3 ± 10.3	25.4 ± 11.6	NS
Log E_2 (pg/ml)	0.302 ± 0.319	0.330 ± 0.280	0.307 ± 0.357	NS
Log T (ng/dl)	2.001 ± 0.347	2.067 ± 0.269	2.050 ± 0.313	NS
L2-4BMD (110)	0.753 ± 0.135	0.739 ± 0.157	0.729 ± 0.118	NS
LT score (89)	−2.6 ± 1.2	−2.6 ± 1.3	−3.3 ± 0.8	NS

NS not significant

other three polymorphisms (Table 2). There were no differences in age, body mass index, or years since menopause among the three SNP types in the four *CYP19* markers (Table 3).

Discussion

We examined the relationship between aromatase-related genes and vertebral fractures by analyzing *CYP19* gene polymorphisms in Japanese women. Among four markers, no differences were found in serum T and E_2 concentrations in the Japanese postmenopausal women. It is possible that local E_2 concentrations are

more important in local tissues rather than serum levels. Bone cells are able to express aromatase and other enzymes required for estrogen synthesis locally (Janssen et al. 1999; Shouzu and Simpson 1998; Watanabe et al. 2004), and aromatase activity in cultured osteoblasts is quantitatively similar to that in adipose stromal cells (Shouzu and Simpson 1998). Thus, estrogen synthesized in bone cells might be important in postmenopausal bone metabolism.

Eriksson et al. (2009) found that genetic variants of rs2470152 in aromatase are associated with E_2 levels, showing that G alleles were correlated with higher serum E_2 levels and BMD in Swedish men than other alleles. Our results, however, showed that the C allele of rs2470152 is associated with vertebral fractures, a finding that suggests that ethnicity, race, and sex differences might influence the results of SNP studies in osteoporosis. The SNP rs2470152 is located in the region of the I.4 promoter (Bulun and Simpson 1994), and it is interesting that the G→A transition of rs2470152 is likely to alter a potential binding site for the binding protein of the transcription factor cAMP response element. The major reason for the discrepancy between our results and those of the Swedish study may be gender differences. The Swedish study focused only on male cohorts. We could not detect any disequilibrium between rs2470152 and the other three markers viewed in HapMap.

CYP19 SNPs (rs10046) were found to be associated with differences in E_2 levels in the European Prospective Investigation of Cancer-Norfolk (EPIC-Norfolk) cohort study (Dunning et al. 2004). SNP rs10046 explains 1.6% of the variance in the $E_2:T$ ratio; however, this SNP is not associated with breast cancer risk (Dunning et al. 2004). The rs10046, a T/C SNP located in the $3'$ UTR, 19 nucleotides downstream from the translation terminus, has been reported to be associated with increased levels of aromatase mRNA expression in tumors (Gruber et al. 2002). In our study, rs10046 was correlated with neither serum E_2 levels nor vertebral fractures. The *CYP19* genotypes demonstrated higher mRNA levels at the rs1062033 locus in postmenopausal osteoporosis. *CYP19* is regulated in a different manner and in different tissues by a hormonally controlled promoter or adipose stromal cell promoter (Mahendroo et al. 1993; Harada et al. 1993). Genetic polymorphisms of *CYP19* might be involved in other processes, such as mRNA stabilization, transcription enhancement, or the post-translational regulation of expression. Neither SNP 1062033 nor rs4775936 was significantly correlated with either serum E_2 levels or vertebral fractures.

We could not detect lower levels of bioavailable serum E_2 by LC–MS/MS in rs2470152; however, another group has shown differences in E_2 levels as measured by RIA according to *CYP19* genotype in a study that included both premenopausal and postmenopausal women (Somner et al. 2004). The discrepancy between the two studies seems to be due to the assay systems used. Bioavailable estrogen levels in postmenopausal women are more relevant than total estrogen levels, which include E_2 bound by sex hormone-binding globulin (SHBG), for bone metabolism. Despite the absence of differences in estrogen levels among the various genotypes, we found that vertebral fracture rates are associated with the *CYP19* genotype in postmenopausal Japanese women in this study. There is much evidence for the role of aromatase activity in bone homeostasis (Miyaura et al. 2001; Oz et al. 2000), and, as previously described, the pharmacological inhibition of aromatase is also associated

with a decrease in BMD and increased risk of fractures (Eastell and Hannon 2005). This indicates that aromatase in local tissues plays roles, both physiologically and pathologically, in bone metabolism.

In conclusion, we provide statistical evidence that the C allele in rs2470152 of the *CYP19* gene is associated with an increased risk of vertebral fractures in postmenopausal Japanese women. Further studies are necessary to detect functional SNPs that induce differences in bone metabolism. Furthermore, we need more participants to detect differences in E_2 levels based on the *CYP19* SNPs of aromatase genes.

References

Arai S, Miyashiro Y, Shibata Y, Kashiwagi B, Tomaru Y, Kobayashi M, Watanabe Y, Honma S, Suzuki K (2010) New quantification method for estradiol in the prostatic tissues of benign prostatic hyperplasia using liquid chromatography–tandem mass spectrometry. Steroids 75:13–19

Bulun SE, Simpson ER (1994) Competitive reverse transcription polymerase chain reaction analysis indicates that levels of aromatase cytochrome P450 transcripts in adipose tissue of buttocks, thigh and abdomen of women increase with advantage age. J Clin Endocrinol Metab 78:428–432

Dunning AM, Dowsett M, Healey CS, Tee L, Luben NL, Folkerd E, Novik KL, Kelemen L, Ogata S, Pharoah PDP, Easton DF, Day NE, Ponder BAJ (2004) Polymorphism associated with circulating sex hormone levels in postmenopausal women. J Natl Cancer Inst 96:936–945

Eastell R, Hannon R (2005) Long-term effects of aromatase inhibitors on bone. J Steroid Biochem Mol Biol 95:151–154

Enjuanes A, Garcia-Giralt N, Supervia A, Nogues X, Ruiz-Gasps S, Bustamante M, Mellibovsky L, Grinverg D, Balcells S, Diez-Perez A (2006) A new SNP in a negative regulatory region of the *CYP19*A1 gene is associated with lumbar spine BMD in postmenopausal women. Bone 38:738–743

Eriksson AL, Lorentzon M, Vandenput L, Labrie F, Linderson M, Syvanen A, Orwoll ES, Cummings SR, Zmuda JM, Ljuggren O, Karlsson MK, Mellstrom D, Ohlsson C (2009) Genetic variations in sex steroid-related genes as predictors of serum estrogen levels in men. J Clin Endocrinol Metab 94:1033–1041

Gruber CJ, Tschugguel W, Schneeberger C, Huber JC (2002) Mechanisms of disease: production and actions of estrogens. N Engl J Med 346:340–352

Haiman CH, Dossus L, Setiawan VW, Stram DO, Dunning AM, Thomas G, Thun MJ, Albanes D, Altshuler D, Ardanaz E, Boeing H, Buring J, Burtt N, Calle EE, Chanock S, Clavel-Chapelon F, Colditz GA, Cox DG, Freigelson HS, Hankinson SE, Hayes RB, Henderson BE, Hirshhorn JN, Hoover R, Hunter DJ, Kaaks R, Kolonel LN, Marchand LL, Lenner P, Lund E, Panico S, Peeters PH, Pike MC, Riboli E, Tjonnelland A, Travis R, Trichopoulos D, Wacholder S, Ziegler (2007) Genetic variation at the *CYP19A1* locus predicts circulating estrogen levels but not breast cancer risk in postmenopausal women. 67:1893-1897

Harada N, Utsumi T, Takagi Y (1993) Tissue-specific expression of the human aromatase cytochrome P-450 gene by alternative use of multiple exons 1 and promoters, and switching of tissue-specific exons 1 in carcinogenesis. Proc Natl Acad Sci USA 90:11312–11316

Hong X, Hsu Y, Terwedow H, Arguelles LM, Tang G, Liu X, Zhang S, Xu X (2007) *CYP19A1* polymorphisms are associated with bone mineral density in Chinese men. Hum Genet 121:491–500

Janssen JMMF, Bland R, Hewison M, Coughtrie MWH, Sharp S, Arts J, Pols HAP, van Leeuwen JPTM (1999) Estradiol formation by human osteoblasts via multiple pathways: Relation with osteoblast function. J Cell Biochem 75:528–537

Mahendroo MS, Mendelson CR, Simpson ER (1993) Tissue-specific and hormonally controlled alternative promoters regulate aromatase cytochrome P450 gene expression in human adipose tissue. J Biol Chem 268:19463–19470

Masi L, Becherini L, Gennari L, Amedei A, Colli E, Falchetti A, Farci M, Silvestri S, Gonnelli S, Brandi ML (2001) Polymorphism of the aromatase gene in postmenopausal Italian women: distribution and correlation with bone mass and fracture risk. J Clin Endocrinol Metab 86:2263–2269

Miyaura C, Toda K, Inada M, Ohshiba T, Matsumoto C, Okada T, Ito M, Shizuta Y, Ito A (2001) Sex and age-related response to aromatase deficiency in bone. Biochem Biophys Res Commun 280: 1062–1068

Olson SH, Bandera EV, Orlow I (2007) Variants in estrogen biosynthesis genes, sex steroid hormone levels, and endometrial cancer: a HuGE review. Am J Epidem 165:235–245

Oz OK, Zerwekh JE, Fisher C, Graves K, Nanu L, Millsaps R, Simpson ER (2000) Bone has a sexually dimorphic response to aromatase deficiency. J Bone Miner Res 15:507–514

Rinancho JA, Zarrabeitia MT, Valero C, Sanudo C, Hernandez JL, Amado JA, Zarabeitia A, Gonzalez-Macias J (2005) Aromatase gene and osteoporosis relationship of ten polymorphic loci withbone mineral density. Bone 36:917–925

Shouzu M, Simpson ER (1998) Aromatase expression of human osteoblast-like cells. Mol Cell Endocrinol 139:117–129

Somner J, McLellan S, Cheung J, Mak YT, Frost ML, Knapp KM, Wierzbicki AS, Wheeler M, Fogelman I, Ralston SH, Hampson GN (2004) Polymorphisms in the P450c17 (17-hydroxylase/17, 20-Lyase) and P450 c19 (aromatase) genes: association with serum sex steroid concentrations and bone mineral density in postmenopausal women. J Clin Endocrinol Metab 89:344–351

Sowers MR, Wilson AL, Kardia SR, Chu J, Ferrell R (2006) Aromatase gene (CYP19) polymorphisms and endogenous androgen concentrations in a multiracial/multiethnic, multisite study of women at midlife. Am J Med 119:S23–S30

Watanabe M, Simpson ER, Pathirage N, Nakajin S, Clyne CD (2004) Aromatase expression in the human fetal osteoblastic cell line SV-HFO. J Mol Endocrinol 32:533–545