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Genetic Polymorphism at Val⁸⁰ (rs700518) of the CYP19A1 Gene is Associated with Aromatase Inhibitor Associated Bone Loss in Women with ER (+) Breast Cancer

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Abstract

Purpose—Polymorphisms in the CYP19A1 (aromatase) gene have been reported to influence disease-free survival and the incidence of musculoskeletal complaints in patients taking aromatase inhibitors (AIs) for estrogen receptor positive (ER+) breast cancer. Bone loss and fractures are wellrecognized complications from AI therapy. The objective of this study is to determine the influence of polymorphisms in the CYP19A1 gene on bone loss among patients taking aromatase inhibitors for ER+ breast cancer.

Patients and Methods—The subjects consisted of 97 postmenopausal women with ER+ breast cancer who were initiated on third-generation AIs. Bone mineral density (BMD) was measured by dual energy x-ray absorptiometry at baseline and at 6 and 12 months. Twenty-four hour urine N-telopeptide (NTX) was measured by Elisa and serum estradiol was measured by ultrasensitive radioimmunoassay at baseline, and at 6 months. Genotyping was done by Taqman SNP allelic discrimination assay.

Results—Women with the AA genotype for the rs700518 (G/A at Val⁸⁰) developed significant bone loss at the lumbar spine and the total hip at 12 months relative to patients carrying the G allele (GA/GG); both $p=0.03$. There was a borderline greater increase in urinary NTX in those with the AA genotype compared to patients with the G allele, $p=0.05$; but no significant difference in changes in estradiol levels among the genotypes.

Conclusion—Patients with the AA genotype for the rs700518 polymorphism in the CYP19A1 gene are at risk for AI-associated bone loss and deserve close follow-up during long-term AI therapy.

Keywords

aromatase inhibitors; CYP19A1; osteoporosis; breast cancer

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INTRODUCTION

For the past few years aromatase inhibitors (AIs) has replaced tamoxifen as the first line agent for endocrine therapy in women with estrogen receptor positive (ER+) breast cancer, either as an adjuvant therapy in early breast cancer (1–4) or as treatment for advanced stage disease (5–7). The use of AIs were shown to result in better disease-free survival, longer time to recurrence and time to metastasis, significantly lesser number of women going into recurrence and better prevention of contralateral breast involvement compared to tamoxifen (1–4).

Aromatase is the enzyme that converts adrenal androgen, androstenedione, to estrone which is the main form of estrogen in postmenopausal women (8). In women with ovarian failure, this process may represent the main if not the only source of estrogen production. Since this remaining estrogen production is important to bone health in postmenopausal women (9;10), inhibition of this pathway may lead to a more profound estrogen deficiency resulting in additional bone loss than what is usually observed in normal menopause. In fact, one of the major concerns in the use of AIs is their effect on bone metabolism, i.e. on bone loss and increase rate of fractures (2;3), which may impact quality of life, as more of these women are surviving breast cancer. For instance, in the Anastrozole, Tamoxifen Alone or in Combination Trial, 11% of the women randomized to anastrozole experienced a fracture compared to 7.7.% in the tamoxifen group after a median followup of 68 months (3). The Breast International Group (BIG) 1–98 trial compared adjuvant therapy with letrozole to tamoxifen. As with the ATAC trial, an increase in the incidence of bone fracture was seen in patients on AIs (11);(12). The effect of AIs on bone metabolism appears to be a class effect for these drugs as bone loss has been observed in both steroidal (exemestane) (4;13) and non-steroidal (letrozole or anastrozole) AIs (3;4;14) in most clinical trials.

As in any form of drug therapy, the response to and susceptibility to side effects from AIs vary between individuals and not all of the AI-treated patients have improvement in survival or experience side effects with AIs (15). This variable response may be explained on the basis of genetic differences among individuals, in particular, genetic variations in the aromatase or CYP19A1 gene.

The CYP19A1 gene which is located on chromosome 15q21.2 and spans 10 exons of which exons 2 to 10 get transcribed and translated to synthesize the aromatase enzyme (16). Several polymorphisms of this gene either positively or negatively correlate with differences in aromatase activity, sex hormone levels, bone mineral density (BMD) and fractures, and response to hormone replacement therapy (17–23). It is possible that these same polymorphisms may also influence susceptibility to the skeletal side effects from AIs leading to significant differences in the degree of bone loss among the variants. Therefore, the aim of this study is to test the hypothesis that skeletal response to AIs is determined by polymorphisms in the CYP19A1 gene. In his study we focused on polymorphisms previously found to be associated with differences in estradiol levels, BMD and fractures.

PATIENTS AND METHODS

Study Population

This is a longitudinal prospective observational study of bone loss among patients taking AIs. Our study population consisted of community-dwelling postmenopausal subjects with ER+ breast cancer, living in the St. Louis, MO, USA, and metropolitan area. Most of the subjects were referred from their oncologist's office. This study was conducted in accordance with the guidelines in the Declaration of Helsinki for the appropriate treatment of human subjects. The protocol was approved by the Washington University School of

Medicine Institutional Review Board and written informed consent was obtained from each participant. These subjects were postmenopausal for at least 12 months from the last menstrual period or have history of bilateral oophorectomy. They should have Stage 0-IIIa ER+ breast cancer who were about to initiate, or within one month from the initiation of a third generation aromatase inhibitor (anastrozole, letrozole or exemestane). Those who were taking medications (estrogen, bisphosphonates, selective estrogen receptor modulators, GnRH analogs, glucocorticoids [5 mg daily of prednisone or equivalent for 1 month] and phenytoin) or had conditions which affect bone metabolism (e.g. hyperthyroidism, osteomalacia, chronic liver disease, renal failure, hypercortisolism, malabsorption and alcoholism) were excluded from the study. Those who were treated with tamoxifen but changed to aromatase inhibitors at the time of enrollment were allowed into the study. Although current smoking was an exclusion criterion, those who resumed or started smoking after they were enrolled in the study were allowed to continue participation.

Clinical, dietary, and anthropometric data

All women were advised to take 1200 mg of calcium and 800 units of vitamin D daily. Data on estrogen exposure was collected using different variables such as age at menarche, number of years of birth control pill (BCP) use, total number of pregnancies, number of pregnancies to term, months of lactation, age at menopause and years since menopause (YSM). Smoking was expressed in pack-years and was estimated as the number of 20-cigarette packs smoked per day multiplied by the number of years of smoking. Body mass index (BMI) was calculated as weight in kilograms divided by square of height in meters (kg/m^2).

Biochemical data

Serum samples were collected in a nonfasting state. Serum estradiol was measured by an ultrasensitive radioimmunoassay technique (Diagnostic Systems Laboratory, Webster, TX, USA). Urinary N-telopeptide of type 1 collagen (NTx) was measured on a 24-h urine sample by an enzyme-linked immunosorbent assay (Osteomark NTx; Ostex International, Seattle, WA, USA) and expressed as nanomoles of bone collagen equivalent (BCE) per millimolar of creatinine. The coefficient of variability for these assays in our laboratory is <10%.

Bone mineral density (BMD)

BMD measurements were performed at baseline, 6 months and at 12 months. The BMD of lumbar spine and proximal femur were measured by dual energy x-ray absorptiometry using a Hologic QDR 4500 (Hologic, Inc., Waltham MA). BMD of the lumbar spine was determined using the antero-posterior projection and was calculated as the average of L1–L4 vertebrae. The nondominant hip was used for proximal femur scans, and values were calculated on the total femur, femoral neck, trochanter, and intertrochanteric areas. BMD values were expressed as grams per square centimeter. The coefficient of variability of this technique using the QDR 4500 densitometer is 1.09% for lumbar spine and 1.2% for total femur in our center.

Genotyping for CYP19A1 gene polymorphisms

Genomic DNA was extracted from peripheral leukocytes using the Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, MN, USA) and used as a template for genotyping procedures.

For this project we selected the single nucleotide polymorphisms (SNPs) that have been reported to result in differences in estrogen levels, BMD and prevalence of fractures, namely: rs4646 (C/A at the 3'UTR), rs700518 (G/A at Val80 in exon 3) and rs1062033 (C/G

at exon I.2). Genotyping was performed by allelic discrimination using specific TaqMan SNP Genotyping Assays and following the manufacturer's instructions. Briefly, ~15 µl PCR reactions were carried out containing 12.5 µl TaqMan Universal PCR Master Mix (Applied Biosystems, New Jersey, USA), 10–15.0 ng DNA template and 1.25 µL TaqMan® Assay primers and FAM/VIC labeled probes by Applied Biosystems as Assays-by-Design™ (Applied Biosystems, Foster City CA 94404). The assay IDs of selected assays were: C__8234730_1_ (rs4646), C__8794675_30 (rs700518), C__8794680_30(rs1062033).

All assays were performed in 96-well plates including negative template controls. After PCR, end point discrimination of alleles was performed on the ABI Prism 7500 using the Sequence Detection Software (Applied Biosystems, Foster City CA 94404). All genotype call rates for each assay were > 95% Quality Value.

STATISTICAL ANALYSIS—Results were expressed as mean ± SD in the text and tables and mean ± SE in the figure. Changes in BMD were expressed as percent (%) change from baseline at 6 and 12 months. A p value of <0.05 was considered significant. Normality for outcome variables was verified by Shapiro-Wilks test. Group comparisons were done by analysis of variance (ANOVA) for continuous variables (adjusted for BMI and baseline BMD for inter-genotype comparisons in BMD changes); while categorical variables were compared using chi-square analysis. The data were managed using Excel 2003 (Microsoft, Redmond, WA) and were analyzed using SAS version 9.2 (SAS Institute, Inc., Cary, NC, USA)

RESULTS

One hundred sixty-nine women with ER+ breast cancer participated in the study, however data from 68 women were excluded from analysis for the following reasons: 36 did not come for a follow-up visit, 23 were on aromatase inhibitors for more than 1 month prior to enrollment into the study, 2 women continued to be on tamoxifen after enrollment, 3 women were started on bisphosphonates, 1 participant did not take aromatase inhibitors, 1 woman was premenopausal and 2 women withdrew consent, leaving data from 101 women, aged 37 to 86 y.o., available for analysis. Of these, 81 women were Caucasians, 19 were African-Americans and 1 one was Asian. There were 3 women who admitted to either continuing to smoke or resumed smoking while in the study.

Table 1 shows that baseline characteristics of the study population. As expected, average estradiol level is in the postmenopausal range. Patients were also in general overweight with low average vitamin D level. Ninety-eight patients had follow-up BMD measurement at 6 months and 82 patients at 12 months. Three patients did not have BMD at six months but did report for BMD measurement at 12 months.

There were no significant differences in phenotypes according to the rs4646 and rs1062033 genotypes in any of the outcomes measured, thus, the results presented below will concentrate only on the analysis according to the rs700518 polymorphism. Ninety-seven women were successfully genotyped for rs700518; 26 women had AA, 43 had AG and 28 had GG genotypes. There was a significant difference in genotype distribution between Caucasians and African-Americans. Among Caucasians, the genotype frequencies were: 17 AA, 37 AG and 26 GG, while genotype frequencies among African-Americans were: 9 AA, 6 AG and 2 GG indicating that in this racial group the AA genotype is the most common genotype compared to AG among Caucasians. One Caucasian woman, 2 African-American women and the only Asian woman participant in the study were either not successfully genotyped or their DNA was not suitable for genotyping. The genotype frequencies

calculated for this polymorphism were in agreement with the Hardy-Weinberg Equilibrium (<http://krunch.med.yale.edu/hwsim/>).

There were no significant differences in baseline clinical features of the participants according to genotypes of the rs700518, except for a significantly higher body mass index in women with the AA genotype compared to patients with the G allele (AG+GG genotypes) (Table 2).

Compared to baseline, there was a minimal and nonsignificant decrease in BMD at the lumbar spine, total hip and femoral neck at 6 months (-1.3 ± 3.2 , -0.41 ± 3.0 and $-1.4 \pm 4.0\%$, respectively) and at one year (-2.1 ± 3.2 , -0.8 ± 3.9 , and $-2.8 \pm 4.8\%$, respectively) in the entire study population. However analysis according to genotypes showed that women with the AA genotype for the G/A base change at Valine 80 for rs700518 had a greater reduction in BMD at the lumbar spine (Fig. 1, **Panel A**) and at the total hip (Fig. 1, **Panel B**) both which were significant at 1 year relative to those carrying the G allele (AG+GG genotype).

There was a significant decrease in the 6-month mean estradiol levels from baseline with AI therapy (13.0 ± 0.70 vs. 8.9 ± 0.72 pg/ml, $p < 0.001$) in the entire population. However, analysis according to the genotypes of the rs700518, showed no significant differences in the degree of reduction in estradiol levels among the variants. There was a trend for an increase in urinary NTX at 6 months compared to baseline from 88.07 ± 116.33 to 141.41 to 166.12 Nmol BCE/mmol creatinine, $p = 0.08$ in a subset of 55 patients who was able to provide a 24-hour urine sample at baseline and at 6 months. Analysis according to genotype for the rs700518 showed that women with the AA genotype had a borderline greater increase in urinary NTX of $77.6 \pm 117.2\%$, compared to women with the AG+GG genotype who had an increase of $27.0 \pm 68.6\%$, $p = 0.052$.

DISCUSSION

The G/A base change at VAL⁸⁰ on exon 3 (rs700518) of the CYP19A1 gene was previously reported to be associated with differences in BMD, estradiol levels and fractures (18;20;24;25). Prior studies indicate that subjects with the A allele for this polymorphism had lower bone densities and lower estradiol levels and may be associated with an increased risk for fracture as part of a common haplotype (20;25). Our results showed that although bone loss in the entire population may not be significant as a group, there is a subset that may be predisposed to bone loss from AI more than others. We found that women with the AA genotype experienced significant AI-induced bone loss compared to subjects with the G allele (AG+GG) suggesting that this group of women may be predisposed to osteoporosis with long-term AI therapy.

The widespread use of AIs in the treatment of ER+ breast cancer has raised some concerns on the musculoskeletal side effects of this class of agents (3;11;13;15). The profound estrogen deficiency created by AIs is assumed to account for the increased bone loss, fractures and incidence of musculoskeletal pains associated with their use. However, not everybody develops these side effects suggesting that perhaps some individuals may be more predisposed to the negative skeletal effects of AIs than others (15).

The aromatase enzyme, which is encoded by the CYP19A1 gene, converts androgen precursors to estrone (the main form of estrogen in postmenopausal women) which is in turn converted to estradiol (26;27). This enzyme assumes a critical role in women after the cessation of ovarian function when adrenal androgens take over as the primary source of estrogen. Any alteration in the activity of this enzyme would theoretically lead to an alteration in circulating estradiol levels (19;21).

The effects of genetic polymorphisms in the CYP19A1 gene on human diseases have been described mostly in the context of hormone-related disorders such as breast cancer (28–30), prostate cancer (31–33) and osteoporosis (17;19–22). For instance, the tetranucleotide simple tandem (TTTA)_n repeat in intron 4 was associated with higher spine BMD in postmenopausal Italian women with more than 11 repeats (17). The converse was true for women with fewer number of repeats who were found to have significantly higher number of incident vertebral fractures. This same polymorphism was also found to influence the rate of bone loss in elderly Italian men (19). Men with more than nine repeats had higher lumbar spine BMD, higher estradiol levels, lower bone turnover markers, and more importantly, lower rates of BMD change than men with low-repeat genotype. Furthermore, in vitro studies confirmed a higher aromatase activity in subjects with the high-repeat genotype. The G to A transversion at Val⁸⁰ in exon 3 (rs700518) was reported to result in significantly lower estradiol and increased prevalence of osteoporosis and fractures in postmenopausal women of Anglo-Saxon origin carrying the G allele (AG+GG) relative to those with the AA genotype (18). Their findings, however, were opposite that found by the group of Lorentzon et. al. in young adult males (18 to 20 y.o.) of Swedish origin from the Gothenburg Osteoporosis and Obesity Determinant (GOOD) study. These investigators reported that males homozygous for the G allele had the highest areal BMD and the greatest cortical bone size compared to carriers of the A allele (24). Although technical problems could be responsible for these variable findings, it is also possible that this discrepancy could be secondary to the gender and the racial background of the population under investigation. Our own data in postmenopausal Caucasian women seemed to agree with the findings from the GOOD study (25). However, the effect of these polymorphisms on bone loss among patients taking AIs, to our knowledge, remains unknown.

Among the 3 SNPs examined, the rs700518 resulted in differential bone loss among the genotypes showing that women with the AA genotype are at risk for AI-associated bone loss. They also experienced a greater increase in NTX levels suggesting an enhanced bone resorption resulting in significant bone loss in 12 months. In a previous work in otherwise healthy postmenopausal women, we reported that women with the A allele (AA+AG) have significantly lower BMD relative to patients with the GG genotype (25). Although there were no significant differences in estradiol levels among the different genotypes, a significant correlation was found between estradiol levels and BMD in women with the A allele but not in women with the GG genotype suggesting that perhaps women with the A allele could be more sensitive to hormonal manipulation procedures. Our findings in the current study confirmed that the skeleton of patients with the AA genotype are perhaps more sensitive to estrogen deprivation from AIs than other genotypes resulting in significant bone loss.

In-vitro studies indicate that some of these polymorphisms are functional (19;21;28). The T allele for the T/C base change in the 3'UTR (rs10046) was found to be associated with a high estrogen profile which correlates with the amount of tumor aromatase mRNA levels in breast tissues (28). This allele is more frequent in breast cancer cases than controls, and women homozygous for this allele had the greatest increase in BMD in response to hormone replacement therapy in postmenopausal women (23). Gene expression studies from fat biopsies and transfection assays of human osteoblastic cells using constructs of another SNP, the rs1062033 (C/G at exon I.2), showed significantly higher aromatase gene expression and transcriptional activity respectively, for the G allele relative to the C allele (21). Clinically, women with the CC genotype had significantly lower BMD and increased vertebral fractures relative to other genotypes. Haplotype analysis showed that rs1062033 and rs700518 belong to the same haplotype with a strong linkage between the 2 SNPs, and may explain the effect of rs700518 on BMD and fracture risk observed in prior studies (20) and the findings in our study

In the current study, although subjects with the AA genotype were experiencing more bone loss than other genotypes, there was no significant difference in the changes in estradiol levels. This observation, however, is not surprising. Whereas peripheral conversion of androgens to estrogens occurs primarily in the adipose tissues accounting mostly for the circulating estrogen levels in postmenopausal women (8), aromatase activity is also present in the osteoblasts providing a local source of estrogen in the bone microenvironment (34). Thus, it is possible that inhibition of the local aromatase activity and estrogen production in the bone tissue may play a main role in bone homeostasis in patients on AIs, independent of circulating estrogen levels.

Our results also showed that women with the AA genotype had significantly higher BMI compared to those with the AG+GG genotypes. This is not surprising as other investigators reported the same findings for this polymorphism (35). Meanwhile other SNPs in the CYP19A1 gene had also been described to be associated with differences in BMI (36;37). Although there is no clear explanation for these observations, estradiol has been reported to stimulate preadipocyte proliferation and differentiation, while testosterone appears to inhibit them (38). In our study there was no significant difference in circulating estradiol level to account for the differences in BMI among the genotypes, but the local concentration in the adipose tissues may be different. It is possible that differences in BMI may have influenced the intergenotype differences in bone loss in our patients, however, analyses on BMD changes in this study are adjusted for differences in BMI indicating that the effect of rs700518 polymorphism on AI-induced bone loss is independent of BMI.

Recent reports suggest that polymorphisms in the CYP19A1 gene influence response to and side effects from aromatase inhibitors. A base change in the 3'UTR (the rs4646) has been reported to result in significant differences on disease-free survival with letrozole therapy among the genotypes in 2 separate studies in women of Spanish ancestry (39;40). In addition the tetranucleotide tandem repeat (TTTA)_n polymorphism was found to be associated with a significant, over twofold (odds ratio [OR]: 0.41; 95% CI: 0.21–0.79; $p = 0.008$), reduced risk of AI-associated arthralgia in women carrying at least one eight-repeat allele (41). Two functional SNPs in the 5'-flanking region (rs6493497 and rs7176005) showed significant differences in the degree of inhibition in aromatase activity with AI therapy (42) which may perhaps lead to differences in response. Nevertheless, to our knowledge, we are the first to report that a polymorphism in the CYP19A1 gene may influence another musculoskeletal side effect of AIs, i.e. bone loss.

In conclusion our study demonstrates that skeletal response to AIs may be modulated by specific genetic traits. Our results indicate that the AA genotype at Val⁸⁰ of the CYP19A1 gene is associated with significant bone loss at the lumbar spine and the total femur in women with breast cancer given AIs. These women may represent the population of women who may be at risk for further bone loss and perhaps osteoporosis with long-term AI therapy and deserve closer follow-up in terms of bone health. In addition, these women may also represent the subset of patients who may be considered for other forms of therapy most especially if they already have low BMD at baseline.

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Highlights

- Polymorphisms in the CYP19A1 (aromatase) gene influence bone loss in postmenopausal women
- Women with AA genotype for the rs700518 (CYP19A1 gene) experienced bone loss relative to patients carrying the G allele
- Patients with the AA genotype for the rs700518 may deserve close follow-up during long-term AI therapy

Fig. 1. Changes in bone mineral density (%) at the lumbar spine (Panel A) and the total hip (panel B) at 6 and 12 months among the genotypes for the rs700518 (Val80). GA and GG genotypes were combined in the analysis due to identical phenotypes. *Both $P=0.03$ by analysis of covariance adjusted for BMI and baseline BMD. Values are means $\pm$ SE.

Table 1

Baseline characteristics of the study population (n=101)

Age (years)	61.0±9.2
BMI (kg/m ²)	30.6±6.0
Years since menopause	13.9±10.5
Racial distribution	N (%)
Caucasians	81 (80.2)
African-Americans	19 (18.8)
Asian	1 (1)
History of previous tobacco use	30 (29.7%)
Positive family history of osteoporosis	32 (31.7%)
Positive family history of breast cancer	49 (48.5%)
Estradiol (pg/ml)	13.0±5.8
25-hydroxyvitamin D (ng/ml)	24.2±14.3

Values are means ± SD

Table 2

Baseline characteristics of the genotypes for the rs700518

	AA (N=26)	AG+GG (N=71)	*P value
Age	59.5±9.9	61.4±8.9	0.37
BMI	32.9±7.4	30.0±5.4	<0.05
YSM	13.9±12.5	13.8±10.0	0.95
Racial distribution			<0.01
Caucasians	17 (21.3%)	63 (78.7%)	
African-Americans	9 (52.9%)	8 (47.2%)	
History of previous tobacco use	11 (42.3%)	20 (28.2%)	0.16
Pack-years of prior smoking	8.72±12.23	6.74±13.39	0.52
Positive family history of osteoporosis	9 (34.6%)	23 (32.4%)	0.84
Positive family history of breast cancer	12 (46.1%)	34 (47.9%)	0.88
BMD (Z-score)			
Spine	1.65±2.10	1.70±1.57	0.91
Total hip	1.45±1.51	1.03±1.09	0.13
Femoral neck	1.13±2.13	0.76±0.14	0.19
Estradiol (pg/ml)	14.16±8.16	12.65±4.60	0.31
25-hydroxyvitamin D (ng/ml)	21.92±18.20	25.06±12.89	0.36
Urine NTX/creatinine (Nmol BCE/mmol creatinine)	112.7±94.1	127.1±136.7	0.71

Values are means ± SD,

*
P by ANOVA