ORIGINAL ARTICLE

Lack of Association Between Estrogen Receptor-Alpha Single-Nucleotide Polymorphism (Codon 594 G-->A) and Postmenopausal Osteoporosis: A Pilot Study

Eva Kassi, MD, PhD,[1] Eirini Soule, PhD,[1] Antonis Kominakis, PhD,[2] Eliana Spilioti, PhD,[1] Paraskevi Moutsatsou, PhD[1]

[1]Department of Biological Chemistry, Medical School, University of Athens, Greece
[2]Department of Animal Breeding, Agriculture University of Athens, Athens, Greece

ABBREVIATIONS
BMD = bone mineral density
DEXA = dual energy X-ray absorptiometry
ERα = estrogen receptor alpha
HWE = Hardy-Weinberg equilibrium
PCR = polymerase chain reaction
PCR-RFLP = PCR-restriction fragment length polymorphism
SD = standard deviation
SE = standard error

Correspondence:
Dr Eva Kassi, Assistant Professor,
Department of Biological Chemistry,
Medical School, Athens University,
75 Mikras Asias Str., Goudi 11527,
Athens, Greece
E-mail: ekassi@med.uoa.gr

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ABSTRACT

BACKGROUND: Recent studies have suggested that the estrogen receptor alpha (ERα) gene is implicated in reduced bone mineral density (BMD).

OBJECTIVE: In the present study, we investigated the relationship between genetic polymorphism in ERα 2014G-->A (T594T) (codon 594 G-->A) and osteoporosis in postmenopausal women.

METHODS: A total of 59 postmenopausal women were included in the study (21 normal, 24 osteoporotic, 14 osteopenic). The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method was used to identify the ERα 2014G->A polymorphism.

RESULTS: The three groups were found to be in genetic equilibrium. Also, there were no allele (p=0.578) and/or genotype frequencies (p=0.59) among the groups, i.e. the three groups could be treated as a genetically uniform population. As expected, normal women exhibited the highest BMD values (1.11 g/cm²) followed by the osteopenic (0.91 g/cm²) and the osteoporotic women (0.73 g/cm²). There was significant difference (p<0.05) in age only between the osteoporotic group (mean age 48.7 years) and either the normal (mean age 46 years) or the osteopenic group (mean age 45 years). No allele and/or genotype effect on BMD or age was detected.

CONCLUSION: Our results support the lack of association between ERα codon 594 and BMD in postmenopausal women, since we found no allele and/or genotype effect on BMD or age. Further studies in a larger sample of postmenopausal women are needed to confirm our results.

INTRODUCTION

Recent studies have suggested that the estrogen receptor alpha (*ERα*) gene is implicated in reduced bone mineral density (BMD). PvuII/XbaI polymorphisms located in

the first intron of the *ERα* gene have been shown to contribute to the determination of BMD in postmenopausal women.[1] In our previous study, we investigated possible genetic alterations in exons 1 and 2 of *ERα* and its association to osteoporosis and familial osteoporosis in women and we found that the codon 10 and codon 87 polymorphisms were not correlated with BMD and bone turnover markers.[2]

In the present study, we focused on genetic alterations of the ligand binding domain (LBD) of ERα and particularly on the relationship, if any, between genetic polymorphism in ERα 2014G>A (T594T) and osteoporosis in postmenopausal women.

MATERIALS AND METHODS

SUBJECTS

The population studied was all of Greek origin. They were divided into the following three groups: (1) 21 normal unrelated women aged 45.9±2.0 years, of postmenopausal status (1 year after last menstrual period); (2) 24 unrelated women who were diagnosed as suffering from idiopathic postmenopausal osteoporosis, aged 48.7±2.7 years; and (3) 14 osteopenic subjects aged 45.0±2.8 years, who were also postmenopausal. The subjects were consecutive outpatients who visited the clinic for clinical evaluation and bone densitometry, at the endocrinology department of Laiko hospital in Athens. The study was developed in agreement with the policy statement of the Declaration of Helsinki and performed with the approval of the Committee on Biomedical Ethics of the University of Athens (Greece). Patients were defined as osteoporotic if they had a T-score less than -2.5 SD and osteopenic with T score values less than -1.0 and ≥2.5 SD.

Detailed medical histories were obtained from each participant and none of the women reported hyperthyroidism, or endogenous/exogenous hypercortisolism, diabetes or any other systemic illness known to affect BMD. They also underwent clinical and biochemical evaluation to exclude other secondary causes of osteoporosis.

BONE MINERAL DENSITY STUDY

Bone mineral density (g/cm^2) was measured at the lumbar spine (L2–L4) by dual energy X-ray absorptiometry (DEXA). The measurements were made using a Norland Xr 26 (Norland Medical Systems Inc., White Plains, N.Y., USA) instrument, with standards from a Greek population. T scores and age-matched comparisons (Z scores) were also calculated using the installed software of the apparatus.

SAMPLE COLLECTION AND DETERMINATION OF ERA G2014A (T594T)

Venous blood (5 mL) from 59 patients was collected and DNA was prepared as previously described. Oligonucleotide primers (forward: 5′-CGCCCGCCG CGCCCCGCGCCCG-GCCCGCCGCCCCCGCCCGCTGTGTCTTCCCACCTA-CAG-3′, reverse: 5′-CGTGTGGGAGCCAGGGAGCT- 3′) were prepared to amplify the coding region of exon 8 manually, as previously described.[3]

Polymerase chain reaction (PCR) product was digested with restriction endonuclease BtgI (PCR- RFLPs: Restriction fragment length polymorphism). The ERα genotype of the DNA samples was identified by electrophoresis in 3 % agarose gels. The ACA/ACA genotype appeared as 227bp bands on agarose gel electrophoresis, ACG/ACG appeared as two bands of 129bp and 98bp and ACG/ACA appeared on all these bands. At least 10 % of the samples belonging to unselected samples was studied twice in order to exclude the digestion errors.

STATISTICAL ANALYSIS

First, the allele frequencies per group were estimated. Standard errors (SE) of the allele frequencies were also calculated, assuming a binomial distribution, as SE(p)=sqrt(pq/2N) where p, q is the frequency of alleles ACG και ACA, respectively and N the sample size. Examination for Hardy-Weinberg equilibrium (HWE) followed using the Genepop software.[4] Age, as well as BMD were subjected to ANOVA fitting alleles or genotypes as the only fixed effect. During BMD analysis, age was additionally fitted as a covariate. Results of ANOVA are presented as least squares means (with SEs). During multiple means contrasts, the Bonferroni correction for p-values adjustment was employed. All these analyses were carried out by SAS (ver 9.0) software.

RESULTS

According to the HWE test the three groups were found to be in genetic equilibrium (Table 1). In addition, there were no allele (p=0.578) and/or genotype frequencies (p=0.59) between groups (both cases, G-like test). This would mean that the three groups could be treated as a genetically uniform population. Table 2 shows the genotype distribution per group (normal, osteopenic and osteoporotic). Table 3 shows the least squares means of age and BMD per group. As it was expected, the normal women exhibited the highest BMD values (1.11 g/cm^2) followed by the osteopenic (0.91 g/cm^2) and the osteoporotic women (0.73 g/cm^2). There was significant difference (p<0.05) in age only between the osteoporotic group (mean age 48.7 years) and either the normal (mean age 46 years) or the osteopenic group (mean age 45 years) (Table 3). Table 4 shows the least squares means (± SE) of BMD and age per allele and genotype. As it is shown, we detected no allele and/ or genotype effect on BMD or age.

TABLE 1. Allele frequency per group and p-value of the test for Hardy-Weinberg equilibrium

2N	Group	Alleles				p-value
		ACG	n	ACA	n	
42	Normal	0.79 ± 0.06	33	0.21 ± 0.06	9	0.195
48	Osteoporotic	0.83 ± 0.05	40	0.17 ± 0.05	8	0.09
28	Osteopenic	0.89 ± 0.06	25	0.11 ± 0.06	3	0.111

n: number of alleles.

TABLE 2. Genotype distribution per group

Group	Genotypes			
	ACG/ACG	ACG/ACA	ACA/ACA	Total
Normal	14	5	2	21
Osteoporotic	18	4	2	24
Osteopenic	12	1	1	14
Total	44	10	5	59

TABLE 3. Least squares means (± SE) of age and bone mineral density (BMD) per group

Group	n	Age (years)	BMD (gr/cm^2)
Normal	21	45.9$_b$ ± 2.0	1.11 ± 0.02
Osteoporotic	24	48.7$_a$ ± 2.7	0.73 ± 0.02
Osteopenic	14	45.0 ± 2.8	0.91 ± 0.03

Means per group with different superscripts are significantly different (p<0.05).

TABLE 4. Least squares means (± SE) of BMD and age per allele and genotype

Allele	n	BMD (gr/cm^2)	Age (years)	Genotype	n	BMD (gr/cm^2)	Age (years)
ACG	87	0.90 ± 0.02	47.9 ± 1,1	ACA/ACA	5	0.90 ± 0.08	43.8 ± 2.3
ACA	19	0.90 ± 0.04	43.8 ± 2,5	ACG/ACA	9	0.89 ± 0.06	43.9 ± 3.6
				ACG/ACG	39	0.91 ± 0,.	48.3 ± 1.7

BMD = bone mineral density.

DISCUSSION

Estrogen seems to play an important role in maintaining normal bone turnover. The estrogen receptor alpha (ERα) gene appears to be the major estrogen receptor mediating estrogen action in all the three types of bone cells and in addition to several other genes has been implicated as a candidate in the genetic basis of osteoporosis.[5] The human *ERα* gene is located on chromosome 6p25.1, comprises eight exons, and spans more than 140 kb.

Interestingly, Tural et al investigating recently the association between osteoporosis and ERα 397 T>C polymorphism in postmenopausal women, found no statistically significant difference in the genotype and allele frequencies of patients and controls, however, the ERα CC genotype compared with TT+TC genotypes was found to increase two-fold the risk of osteoporosis.[6] Moreover, Erdogan et al, studying the relationship among bone mineral density (BMD) values of lumbar vertebra and femoral neck and ERα gene PvuII and XbaI polymorphisms in intron 1, found that *ERα* gene *Pvu*II polymorphism was effective on average lumbar vertebra BMD value in postmenopausal women.[7] In the same line, other studies in Korean and Turkish postmenopausal women confirmed that the ER PvuII and XbaI polymorphisms were associated with BMD in lumbar spine and femoral neck.[8,9]

Codon 594 is a silent mutation in the ligand binding domain (AF-2) of ERα, thus it does not alter the protein; however, it may affect gene expression via the sequence change in RNA influencing ERα gene transcription, processing or translation, thus resulting in different reactions to estrogens. Indeed, a change in synonym codons might call for a different tRNA to respond, influencing therefore the efficiency of translation and receptor protein expression.[10] Moreover, the codon 594 polymorphism may be linked with a 'causal' variant in the immediate vicinity of the ERα gene itself or another unidentified gene nearby.

Several studies have been conducted investigating the relationship between codon 594 polymorphism and various diseases. Breast cancer, migraine, knee osteoarthritis, systemic lupus erythematosus and renal cancer have been associated with the presence of codon 594 polymorphism.[11-14] To our knowledge, this is the first study on a possible association between codon 594 polymorphism and postmenopausal osteoporosis.

Although there were age differences among the three groups studied (normal/ osteopenic and osteoporotic), all women were in post-menopausal status. Moreover, no statistical significance in the mean duration of post-menopausal status was observed among the three groups. The main limitation of this preliminary study is the small number of participants leading in a statistical power equal to 0.1.

CONCLUSION

Our results support the lack of association between ERα codon 594 and bone mineral density (BMD) in postmenopausal women, since we found no allele and/or genotype effect on BMD or age. Further studies in larger sample sizes are needed to confirm our results.

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