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ORIGINAL ARTICLE

Osteoporosis and its Association with Estrogen Receptor- alpha Gene Polymorphism in a population of Iranian Women Referring to Loghman Hospital

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Osteoporosis is a common disease in which the bones become prone to fracture as a result of loss of bone mineral density (BMD). The estrogen receptor (ER) gene is a candidate gene for osteoporosis. This study assesses the relation between estrogen receptor-α gene polymorphism and osteoporosis in a population of Iranian women. Materials & Methods: In the present study, we investigated 200 pre- and/or postmenopausal Iranian women, aged 35-80 years, stratified for BMD into normal and patient groups. The genomic DNA of both groups was amplified by PCR using specific primers and products were digested by restriction enzymes PvuII or XbaI to identify the related genotypes. The genotypes of intron 1 PvuII or XbaI polymorphisms of the ER-α gene were detected and introduced so that the upper case and lower case letters of Pp (PvuII) and Xx (XbaI) signified the absence or presence of restriction sites in RFLP experiments. Results: Based on our results, no significant relationship was observed between BMD and intron 1 RFLPs of the estrogen receptor alpha gene. Three genotypes, Pp XX, pp XX and PP xx, were detected, all at a very low frequency in this population of Iranian women. Conclusion: To conclude no significant relationship was found between BMD and intron 1 RFLPs of the estrogen receptor alpha gene. Larger numbers of patients need to be investigated to ascertain and confirm whether ER-α genotypes are associated to the disease etiology and if any other factors are involved.

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Introduction

Osteoporosis is increasingly considered as a major public health problem in the aging populations of most countries worldwide. Bone mineral density (BMD), the main determinant of osteoporosis fracture risk,[1] besides being affected by the environment, is strongly influenced by genetic factors,[2] first reported in the association shown between BMD and vitamin D receptor (VDR) gene

polymorphism.[3] However, there is still disagreement whether the VDR polymorphisms are definitely responsible for loss of BMD.[4] On the other hand, estrogen and its receptors play an important role in controlling skeletal growth and maintenance of bone mass,[5] and estrogen therapy has been shown to prevent bone mineral loss.[6] Moreover, inactivation of the ER-α gene is associated with low BMD, indicating that this gene is a strong candidate for osteoporosis. ER-α belongs to the nuclear receptor super family of ligand-inducible transcription factors[7] and it is also implicated in the development or progression of numerous diseases, which include but are not limited to various types of cancer.[8] ER-α gene, located at 6q25.1, is greater than 140 kb in length and splits into eight exons and seven introns. Several polymorphic sites within the ER-α gene locus have been revealed by genetic screening[9,10] of which the most widely studied are TA dinucleotide repeat polymorphism at the 5' upstream of exon 1 and PvuII and XbaI RFLPs of the intron 1. Although many publications have demonstrated the relationship between ER-α polymorphisms and BMD in different populations,[11-14] their association varies across different countries.[15] The TGFβ1[16] androgen receptor,[17] IGF 1 gene,[18] Interleukin-1 receptor antagonist,[19] Interleukin-6,[20] and the collagen type I alpha 1 gene (COLIA1),[4,21] are among nearly sixty candidate genes discovered so far that have been implicated as determinants of bone mass. The conflicting results might be due to different genetics and environmental background such as diet, exercise and drugs in different cohorts.[11] Clarification of the role of these genes will eventually lead to more advanced diagnostic methods and availability of more efficacious drugs targeting osteoporosis.[22] In the current study, our goal was to examine the role of intron 1 PvuII and XbaI polymorphisms of the ER-α gene in a population of pre- and/or post-menopausal Iranian women, stratified for BMD into normal and patient groups.

Materials and Methods

Subjects: Unrelated women, referred for acute skeletal pain to the rheumatology clinic and the BMD department of Loghman Hospital in Tehran, Iran, underwent dual energy x-ray absorptiometry (DXA); of these, 100 were randomly selected as controls and 100 as cases according to their BMD values. A detailed profile including medical, personal and family history was obtained from all subjects, aged 35-80 years, and any women with a history of using hormonal drugs, calcium tablets or having any dietary habits that would affect bone mass and turnover, were excluded from analysis.

Measurement: For each subject, BMD (g/cm^2) was measured at lumbar spine (L1-4), femoral neck, the trochanter and ward triangle by dual-energy X-ray abrop-tiomerty (DXA; Lunar DPX-L densitometer, Lunar Corp., Madison, WI).

Genotyping: Genomic DNA was extracted and purified from EDTA blood samples (of each volunteer) using the method of Miller et al. 1988 [23]. Genotypic analysis of ER-alpha PvuII, and XbaI gene polymorphisms was done by polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP). The primers were designed to amplify a part of intron 1 and exon 2 of the ER gene. PvuII and XbaI polymorphisms are 45 bp apart and located approximately 400 bp upstream of exon 2.

PCR: Amplification of a 527 bp PCR fragment was performed using 0.1 µg of extracted DNA in 50 µl of buffer solution [1X PCR buffer (Cinnagen, Karaj, Iran), 1.5 mM MgCl2, 100 µM dNTP mix and 39.3 µl DDW] with 1 U of Taq. DNA polymerase (Cinnagen, Karaj, Iran) and 100 nM of each Oligonucleotide primer (Forward primer: 5'ATCCAGGGTTATGTGGCAATGAC3', Reverse primer: 5'ACCCTGGCGTCGATTATCTGA3'). PCR was performed for 40 cycles with the following steps: Denaturizing at 94°C for 30s, annealing at 59°C for 40s and elongation

at 72°C for 1 minutes and a final extension of 2 minutes at 72°C.

Restriction digest: For amplification, samples of 50 µl containing 1x PCR buffer, 1.5 mM MgCL2, 100 mM dNTP Mix, 100 nM each primer, 1 unit of Taq DNA polymerase, 0.1 µg genomic DNA were subjected to 40 cycles of amplification. Each sample was resolved on a 2% agarose gel containing ethidium bromide. The length of the product is 527 bp (lane 2-7). The marker was 100bp DNA ladder (lane 1).

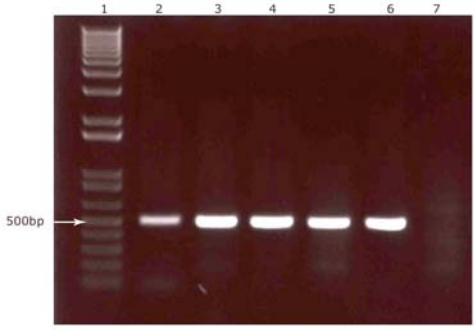

Figure 1. Amplification of ER-alpha gene fragment.

After amplification, the PCR products (fig.1) were digested with 2 IU of either PvuII or XbaI restriction enzymes (New England Biolabs, Ipswich, MA , USA) and resolved on 2.0% agarose gels with ethidium bromide staining (figure 2). The genotypes were represented as Pp (PvuII) and Xx (XbaI), with upper case and lower case letters signifying the absence and presence of the restriction site, respectively. RFLP was performed for each sample separately.Two set of reactions of 30 µl were prepared. Each reaction contained 1x NE buffer, 2 units PvuII or XbaI, 4-6 ng/ µl PCR product, 1X BSA for XbaI digestion reaction. Samples were analyzed on 2% agarose gel. Lane 1 and 6: 100 bp DNA ladder, PvuII digestion results: Lines 2-5, XbaI digestion products: lines 7-10 (figure 2).

Statistical analysis: All statistical analyses were carried out using the SPSS software package (SPSS 10.0.0, Chicago, IL, USA). Changes in BMD were analyzed by non-paired T-scores and Z- scores. Genotype frequencies of controls and patients were compared using the Pearson, Chi-square and Fischer exact tests. A p-value of less than 0.05 was considered statistically significant.

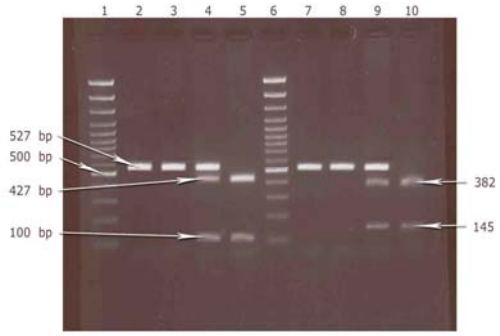

Figure 2. Restriction digest of PCR product.

Results

The frequencies of the ER-α genotypes were almost similar to previously published genotype frequencies in European and East-Asian populations.

Table 1. Comparison of spine and femur bone mass density mean value in the case and control groups according Z and T scores

Groups	(Mean±SD)
SP.Z	
Control	0.411±1.0*
Case	-1.296±843
SP.T	
Control	0.285±1.1
Case	-1.959±0.887
SP.BMD	
Control	1103.33±124.281
Case	846.46±100.483
FEM.Z	
Control	0.741±0.919
Case	0.393±0.873
FEM.T	
Control	0.700±1.146
Case	-0.862±1.02
FEM.BMD	
Control	967.26±112.713
Case	797.53±119.27

SP.Z: SpineZ-score,SP.T: SpineT-score,
SP.BMD: Spine bone mass density,
FEM.Z:FemurZ-score,FEM.T: FemurT-score,
FEM. BMD=Femur bone mass density,
* P<0.001

The study subjects were unrelated and aged 37-70 years with an average spine BMD of -1.296 (Z-score) and -1.959 (T-score), respectively (Table 1). As it is shown in this table, the mean of spine BMD in control group was 0.41 by Z-score and 0.285 with T-score. The difference between the two groups in spine BMD according to T-scores and Z-scores was significant ($p \leq 0.0001$). The ER-alpha genotypes were obtained by PCR (Fig.1) followed by restriction enzyme PVUII and XbaI digestion (Fig. 2).

Interestingly, while the control group showed no osteoporosis of the spine area (T-score$\leq$-2.5), 25% of the patients showed osteoporosis with the same evaluation (Table 2), indicating a significant difference in the percentage of osteoporosis ($p<0.0001$).

Although no significant difference was found between the two groups in the femoral neck ($p \leq 1$, Z-score with Fisher exact test), there was a significant difference in the lumbar spine region ($p \leq .007$, Z-score with Fisher's exact test), Table 2. After performing PCR-RFLP, the two groups showed no sig-nificant difference regarding their P geno-types, PP, Pp, pp, ($p=.471$) or X genotypes, XX, Xx, xx, ($p=.6$), Table 2. Moreover, the spine T-score shows that 12.5% of the 200 people under study were osteoporotic, whereas considering the femoral neck T-score, only 3% of the 200 individuals were osteoporotic. Based on the Z-score, only 4% were osteoporotic in the spine and 5% were osteoporotic in the femoral neck region, respectively.

Table 2. Comparison between P and X genotype frequency of case and control groups, comparing the T and Z- scores for spine and femur of both groups

Groups		Control Numbers (%)	Case Numbers (%)
P-Genotype			
PP		17 (17%)	21(21%)
Pp		51(51%)	53(53%)
pp		32(32%)	26(26%)
X-Genotype			
XX		14(14%)	18(18%)
Xx		76(76%)	70(70%)
Xx		10(10%)	12(12%)
P-X-Genotype			
P- X-		68(68%)	74(74%)
Ppxx, ppXx, ppxx		32(32%)	26(26%)
Tscore for			
Spine	(+)	0	25
Osteoporosis	(-)	100	75
Tscore for			
Femur	(+)	0	6
Osteoporosis	(-)	100	94
Zscore for			
Spine	(+)	0	8
Osteoporosis	(-)	100	92
Zscore for			
Femur	(+)	0	1
Osteoporosis	(-)	100	99

* $P<0.0001$, ** $P<0.05$

Discussion

In this study, as in other similar investigations, we randomly introduced control and patient groups to our study to determine any possible correlation of the ER-alpha gene polymorphism with disease prevalence. The distribution of the PvuII and XbaI RFLPs was as follows: PP 38 ((19%), Pp 104(52%), pp 58(29%) and XX 32(16%) Xx 146(73%), and xx 22(11%), where capital letters denote the 'absence of', and lower case letters 'the presence of' the restriction site of each RFLP. Our results show that the prominent genotypes of our study population, both in controls and patients, are the heterozygote for both bone markers. Although statistical T and Z-scores showed 25% of the patients had low BMD in the spine and the lumbar spine showed a significant difference, as compared to the controls, based on PCR-RFLPs, the PvuII (PP, Pp, pp) and XbaI genotypes (XX, Xx, xx), showed no significant correlation between the disease and the genotypes investigated.

The frequencies of the ER-α genotypes were almost similar to previously published genotype frequencies in European and East-Asian populations. The distribution of PvuII alleles observed in our study did not differ significantly from those detected in 8 European centers,[24] Asian women[25] and a Polish population;[26] however, our distribution was completely different from a Bulgarian population.[27] With regard to the XbaI distribution, compared to other studies, we observed that the distribution of XbaI alleles in our pre- and post menopausal women differed to that of other populations.[24-27] Contrary to data for Caucasians, reporting an increased frequency of the Px haplotype and a reduced frequency of the PX haplotype,[28] we observed a majority of P-X- haplotypes among our population (71%). Indeed, this inconsistency among ER polymorphism studies may be explained by the existence of a differential degree of linkage disequilibrium among different ethnic populations.[29]

Inconsistent associations, between ER1 gene polymorphism and BMD have been reported by several studies among pre- and post-menopausal women. Some studies suggested an association between PvuII and XbaI restriction fragment length polymorphisms (RFLPs) and low BMD,[10] whereas others found a significant association between the PvuII–XbaI haplotype (Px) and decreased BMD.[30] We observed no significant correlation between the PvuII and XbaI genes polymorphism and BMD, a finding consistent with a number of studies.[28-31] Recent studies have demonstrated that PvuII, and XbaI RFLPs were not associated with BMD among Korean and Chinese women.[8,32] Our study does have some potential limitations; differences among our population in terms of ethnic background, age, menopausal status, environment and genetic make-up may have confounded the results of BMD measurement, statistically. Currently, the guideline for diagnosis and management of osteoporosis is changing i.e. patient's bone density needs to be interpreted in the context of age, sex and other risk factors for fracture.[33,34] Although many studies have illustrated that BMD can be considered as a polygenic trait,[35] it has been revealed that baseline BMI and change in menstrual status contributed more to the magnitude of the difference in bone change.[36] A positive association between the gene polymorphism and osteoporosis could open new windows to disease therapy by recognizing the importance of predicting disease susceptibility in high risk women and monitoring them regularly even before menopause.

We conclude that, in this population, PvuII and XbaI polymorphisms at the ER- α gene locus were not found to be the main genetic determinants of bone mass density. Further studies of larger groups will facilitate the evaluation of differences in bone density by genotypes.

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References

1. Kanis JA, Melton LJ, Christiansen C, Johnston CC, Khaltaev N. The diagnosis of osteoporosis. J Bone Miner Res 1994; 9: 1137-41.
2. Richards JB, Rivadeneira F, Inouye M, Pastinen TM, Soranzo N, Wilson SG, et al. Bone mineral density, osteoporosis, and osteoporotic fractures: a genome-wide association study. Lancet 2008; 371: 1505-12.
3. Dundar U, Solak M, Kavuncu V, Ozdemir M, Cakir T, Yildiz H, et al. Evidence of association of Vitamin D receptor Apa I gene polymorphism with bone mineral density in postmenopausal women with osteoporosis. Clin Rheumatol 2009; 28: 1187-91.
4. Uitterlinden AG, Ralston SH, Brandi ML, Carey AH, Grinberg D, Langdahl BL, et al. The association between common vitamin D receptor gene variations and osteoporosis: a participant-level meta-analysis. Ann Intern Med 2006; 145: 255-64.
5. Tranah GJ, Taylor BC, Lui LY, Zmuda JM, Cauley JA, Ensrud KE, et al. Genetic variation in candidate osteoporosis genes, bone mineral density, and fracture risk: the study of osteoporotic fractures. Study of Osteoporotic Fractures (SOF) Research Group. Calcif Tissue Int 2008; 83: 155-66.
6. Lindsay R, Hart DM, Purdie D, Ferguson MM, Clark AS, Kraszewski A. Comparative effects of estrogen and progesterone on bone loss in postmenopausal women. Clin Sci Mol Med 1978; 54:193-5.
7. Achari Y, Lu T, Hart DA. Polymorphisms in the promoter regions for human MMP-1 and MMP-13 lead to differential responses to the alpha and beta isoforms of estrogen receptor and their ligand in vitro.Biochim Biophys Acta 2008; 1782: 391-400.
8. Deroo BJ, Korach KS. Estrogen receptors and human disease. Clin Invest 2006; 116: 561-70.
9. Castagnoli A, Maestri I, Bernardi F, Del senno L. PvuII RFLP inside the human estrogen receptor gene. Nucleic Acids Res 1987; 15: 866.
10. Kastelan D, Grubic Z, Kraljevic I, Polasek O, Dusek T, Stingl K, et al. The role of estrogen receptor-alpha gene TA polymorphism and aromatase gene TTTA polymorphism on peak bone mass attainment in males: is there an additive negative effect of certain allele combinations? J Bone Miner Metab 2009; 27: 198-204.
11. Kobayashi N, Fujino T, Shirogane T, Furuta I, Kobamatsu Y, Yaegashi M, et al. Estrogen receptor alpha polymorphism as a genetic marker for bone loss, vertebral fractures and susceptibility to estrogen. Maturitas 2002; 41: 193-201.
12. Lau EM, Young RP, Lam V, Li M, Woo J. Estrogen receptor gene polymorphism and bone mineral density in postmenopausal Chinese women. Bone 2001; 29: 96-8.
13. Han KO, Moon IG, Kang YS, Chung HY, Min HK, Han IK. Non-association of estrogen receptor genotypes with bone mineral density and estrogen responsiveness to hormone replacement therapy in Korean postmenopausal women. J. Clin Endocrinol Metab 1997; 82: 991-5.
14. Aerssens J, Dequeker J, Peeters J, Breemans S, Broos P, Boonen S. Polymorphisms of the VDR, ER and COLIA1 genes and osteoporotic hip fracture in elderly postmenopausal women. Osteoporos Int 2000; 11: 583-91.
15. Ralston SH. Genetic determinants of osteoporosis. Curr Opin Rheumatol 2005; 17: 475-9.
16. Langdahl BL, Uitterlinden AG, Ralston SH, Trikalinos TA, Balcells S, Brandi ML, et al; GENOMOS Study. Large-scale analysis of association between polymorphisms in the transforming growth factor beta 1 gene (TGFB1) and osteoporosis: the GENOMOS study. Bone 2008; 42:969-81.
17. Sowers M, Willing M, Burns T, Deschenes S, Hollis B, Crutchfield M, et al. Genetic markers, bone mineral density and serum osteocalcin levels. J Bone Miner Res 1999; 14: 1411-9.
18. Rosen CJ, Kurland ES, Vereault D, Adler RA, Rackoff PJ, Crai, et al. Association Be-

tween Serum Insulin Growth Factor-I (IGF-I) and a Simple Sequence Repeat in IGF-I Gene: Implications for Genetic Studies of Bone Mineral Density. J Clin Endocrinol Metab 1998; 83: 2286-90.
19. Keen RW, Woodford-Richens KL, Lanchbury JS, Spector TD. Allelic variation at the interleukin-1 receptor antagonist gene is associated with early postmenopausal bone loss at the spine. Bone 1998; 23: 367-71.
20. Tsukamoto K, Ohta N, Shirai Y, Emi M. A highly polymorphic CA repeat marker at the human interleukin 6 receptor (IL6R) locus. J Hum Genet 1998; 43: 289-90.
21. Lindahl K, Rubin CJ, Brändström H, Karlsson MK, Holmberg A, Ohlsson C, et al. Heterozygosity for a coding SNP in COL1A2 confers a lower BMD and an increased stroke risk. Biochem Biophys Res Commun. 2009; 384: 501-5.
22. Niu T, Xu X. Candidate genes for osteoporosis. Therapeutic implications. Am J Pharmacogenomics 2001; 1: 11-19.
23. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 1988; 16: 1215.
24. Ioannidis JP, Ralston SH, Bennett ST, Brandi ML, Grinberg D, Karassa FB, et al. GENOMOS Study. Differential Genetic Effects of ESR1 Gene Polymorphisms on Osteoporosis Outcomes. JAMA 2004; 292: 2105-14.
25. Rizzoli R, Bonjour JP, Ferrari SL. Osteoporosis, genetics and hormones. J Molec Endocrinol 2001; 26: 79-94.
26. Jakimiuk A, Nowicka M, Bogusiewicz M, Adamiak A, Skorupski P, Miotla P, et al. Prevalence of estrogen receptor PvuII and XbaI polymorphism in population of Polish postmenopausal women. Folia Histochem Cytobiol 2007; 45: 331-8.
27. Ivanova JT, Doukova PB, Boyanov MA, Popivanov PR. PvuII and XbaI polymorphisms of the estrogen receptor gene and bone mineral density in a Bulgarian population sample. Hormones 2007; 6: 36-43.
28. Van Meurs JB, Schuit SC, Weel AE, Van der Klift M, Bergink AP, Arp PP, et al. Association of 5' estrogen receptor alpha gene polymorphisms with bone mineral density, vertebral bone area and fracture risk. Hum Mol Genet 2003; 12:1745-54.
29. Becherini L, Gennari L, Masi L, Mansani R, Massart F, Morelli, A, et al. Evidence of a linkage disequilibrium between polymorphisms in the human estrogen receptor alpha gene and their relationship to bone mass variation in postmenopausal Italian women. Hum Mol Genet 2000; 9: 2043-50.
30. Albagha OM, Pettersson U, Stewart A, Mc Guigan FE, Mac Donald HM, Reid DM, et al. Association of oestrogen receptor alpha gene polymorphisms with postmenopausal bone loss, bone mass, and quantitative ultrasound properties of bone. J Med Genet 2005; 42: 240-6.
31. Langdahl BL, Lokke E, Carstens M, Stenkjaer LL, Eriksen EF. A TA repeat polymorphism in the estrogen receptor gene is associated with osteoporotic fractures but polymorphisms in the first exon and intron are not. J Bone Miner Res 2000; 15: 2222-30.
32. Qin YJ, Zhang ZL, Huang QR, He JM, Hu YQ, Zhao Q, et al. Association of vitamin D receptor and estrogen receptor-alpha gene polymorphism with peak bone mass and bone size in Chinese women. Acta Pharmacol Sin 2004; 25:462-8.
33. Siminoski K, Leslie WD, Frame H, Hodsman A, Josse RG, Khan A, et al. Recommendations for bone mineral density reporting in Canada: a shift to absolute fracture risk assessment. J Clin Densitom 2007; 10: 120-3.
34. Cooper, Mark S. Our approach to osteoporosis screening and treatment needs to change. CMAJ 2008; 178: 1683-4.
35. Johnson J, Wang MY. Genome search reveals gene variants associated with low bone mineral density and fractures in osteoporosis. Neurosurgery 2008; 63: N12.
36. Melton LJ, Atkinson EJ, O'Connor MK, O'Fallon WM, Riggs BL. Determinants of bone loss from the femoral neck in women of different ages. J Bone Miner Res 2000; 15: 24-31.