CLINICAL STUDY

Estrogen receptor α gene polymorphisms (*Pvu* II and *Xba* I) influence association between leptin receptor gene polymorphism (Gln223Arg) and bone mineral density in young men

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Abstract

Objective: The peak bone mass is recognized as an important determinant in the development of osteoporosis. We investigated associations between bone mineral density (BMD) and polymorphisms of the leptin receptor (LEPR) and estrogen receptor α (ER α) genes in young men.

Design: BMD, anthropometric characteristics, and serum leptin concentrations were measured in young men and compared with regard to the LEPR and ER α genotype.

Methods: From 219 healthy volunteers aged 20–34 years, we genotyped the Lys109Arg, Gln223Arg, Ser492Thr, Ala976Asp, and Pro1019Pro variants of LEPR and the *Pvu* II and *Xba* I variants of ER α using the polymerase chain reaction-restriction fragment length polymorphism method. We determined serum leptin concentrations by radioimmunoassay (RIA) and BMD by dual energy X-ray absorptiometry.

Results: The subjects carrying the Gln223 allele of LEPR had higher BMD at the lumbar spine compared with the subjects without this allele. There were no significant differences in BMD among *Pvu* II and *Xba* I genotypes of ER α . However, an association between LEPR and BMD was noted in the subjects carrying the PP homozygotes of *Pvu* II or the X alleles of *Xba* I, but this was not significant in those without these genotypes.

Conclusions: This study indicates that the Gln223Arg polymorphism of LEPR is important for determination of the peak bone mass in men and that it is influenced by ER α gene polymorphisms.

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Introduction

Although osteoporosis has generally been considered to be a disease of women, it is also known to be prevalent in men. The incidence of osteoporotic fracture in men is about half of that in women (1). Furthermore, the mortality associated with hip fracture in elderly men is considerably higher than in women (2).

At a given age, the amount of bone mass is determined by the peak bone mass accrued between 20 and 34 years of age and the subsequent age-related bone loss (3). The peak bone mass is recognized as an important determinant in the development of osteoporosis (4). Although environmental factors such as nutrition and physical activity may affect the peak bone mass, genetic factors are the deciding determinants (5). Several genes, such as the vitamin D

receptor, estrogen receptor α (ER α), type 1 collagen, and transforming growth factor- β genes, have been studied for an association between their genetic polymorphisms and bone mass (6). In most of these studies, however, the study populations have been postmenopausal women, in whom accretion of bone mass has ceased and bone loss has begun. Furthermore, these data have been inconsistent, pointing to the possibility that gene–gene and gene–environment interactions may represent important confounding factors in genetic studies.

Leptin, the product of the *ob* gene, is an adipocyte-derived hormone that plays a pivotal role in body weight homeostasis, neuroendocrine function and fertility (7, 8). Recently, leptin has emerged as a key element in the regulation of bone mass, although the mechanism by which leptin acts upon bone mass

remains controversial (9, 10). The biological actions of leptin on target tissues are carried out through binding to its specific receptor, which is a member of the cytokine receptor family (11). Although the polymorphisms of the leptin receptor (LEPR) gene have been extensively studied for their association with obesity (12–15), there is no information on whether they are associated with bone mass.

Even in men, estrogen has been considered to be more important than testosterone in the maintenance of bone mass (16, 17). Recently, ER α gene polymorphism has been reported to be associated with bone mineral density (BMD) in boys (18). These results propose that the ER α gene polymorphism may influence accumulation of bone mass. However, no subsequent studies that support such an association in men have been reported.

Interestingly, estrogen interacts with leptin in various ways. Estrogen reduces LEPR gene expression, alters the balance between the long and short forms of LEPR, and subsequently regulates the sensitivity to leptin (19). Estrogen elevates mRNA expression of leptin in cultured rat adipocytes (20). In humans, it was reported that hormone replacement therapy significantly elevated plasma leptin levels in postmenopausal women (21), although these results remain controversial (22). Taken together, we hypothesized that genetic variations of ER α , which is known to mediate estrogenic actions, may modify the effect of the leptin system on bone metabolism. However, there has been no study to date on the interactions of these genes in relation to bone mass. This led us to investigate associations between BMD and polymorphisms of the LEPR and ER α genes, and the possible interactive effects of polymorphisms of ER α and LEPR genes on BMD in young men.

Subjects and methods

Subjects

The study population comprised 220 healthy young men who were medical students of the University of Ulsan or residents at a university hospital (Asan Medical Center (AMC)), Seoul, Korea. All of them were Korean and they had volunteered for the study. The study was approved by the AMC ethics review committee and a written informed consent was obtained from all subjects. They completed a self-administered questionnaire concerning demographic characteristics, general health status, medication, weekly duration of weight-bearing physical activity, and their smoking and drinking habits. Daily dietary calcium intake was assessed by administration of a food frequency questionnaire. This was based on Willett's food frequency questionnaire (23), and adapted for the Korean diet (24). It should be noted that the reliability of this semi-quantitative food frequency questionnaire is

limited, since the correlation coefficient comparing this and the nutrient intake estimated by a 3-day diet record was only 0.57 for the assessment of calcium intake (by Pearson's correlation, $P < 0.05$) (24). Of the 220 subjects, one subject who had taken finasteride for alopecia was excluded. The others were free from drugs and diseases known to affect bone metabolism.

Venous sampling and measurement of leptin concentration

After overnight fasting, venous blood samples were collected in EDTA and plain tubes, promptly separated by centrifugation and stored at -76°C until analysis. The serum leptin concentration was determined using a commercial RIA kit (Mediagnost, Aspenhaustrasse, Reutlingen, Germany) with a sensitivity of 0.1 ng/ml.

Bone mineral densitometry

BMD (g/cm^2) of the lumbar spine (L2–L4, anteroposterior) and the left proximal femur (femoral neck, Ward's triangle, trochanter, and shaft) were measured by trained personnel using a Lunar Corp. Expert XL (Lunar, Madison, WI, USA) dual energy X-ray absorptiometer, software version 1.90. The *in vivo* precision of the machine was 0.82% for the lumbar spine and 1.12% for the femur neck.

Genotype analysis with polymerase chain reaction and restriction fragment length polymorphism analysis

Genomic DNA was extracted from peripheral blood leukocytes using a commercial kit (Wizard Genomic DNA purification kit; Promega, Madison, WI, USA). Genotyping of the DNA sequence variants in the Lys109Arg, Gln223Arg, Ser492Thr, Ala976Asp, and Pro1019Pro sites of the LEPR gene and in the PvuII and XbaI sites of the ER α gene was carried out by restriction enzyme analysis of polymerase chain reaction (PCR)-amplified DNA, using the specific pairs of oligonucleotide primers as previously described (15, 25, 26). The PCR primers were sequence-specific and/or mutated for convenience. Amplification of the LEPR and ER α genes was carried out using a thermocycler (Perkin Elmer, Boston, MA, USA) by a method described previously with some modifications (15, 25, 26). After the amplification, the PCR products were digested with the restriction endonucleases *Hae*III, *Msp*I, *Pst*I, *Hae*III, and *Hinc*II (New England Biolabs Inc., Beverly, MA, USA) for the Lys109Arg, Gln223Arg, Ser492Thr, Ala976Asp, Pro1019Pro polymorphisms of the LEPR gene respectively. *Pvu*II and *Xba*I (New England Biolabs Inc.) were used for the ER α gene. The DNA fragments were then electrophoresed on 3% agarose gels for LEPR and 1% agarose gels for ER α .

Statistical analysis

Descriptive characteristics were expressed as the mean and standard deviation. Logarithmic transformation was used for serum leptin levels because of right-skewed deviation. Pearson correlation analysis and multiple linear regression analysis were used to summarize the clinical and laboratory characteristics in the studied subjects.

Genotype distribution of the polymorphisms was tested for Hardy–Weinberg equilibrium by χ^2 test. Linkage disequilibrium resulting from the non-random association of the genotypes was also assessed by χ^2 test.

An unpaired *t*-test or the Mann–Whitney U test was used for the analysis of the differences in age, height, body weight, body mass index (BMI), amount of calcium intake, smoking and drinking habits, time of exercise, and serum leptin levels between the different groups. The impact of the polymorphisms on BMD was evaluated using multiple linear regression analyses with age, BMI and the polymorphisms as independent variables. An interaction between the genotypes was assessed by analysis of covariance. *P* values less than 0.05 were considered significant. SPSS 10.0 package (SPSS Inc., Chicago, IL, USA) was used for statistical procedures.

Results

Subject characteristics

The mean age of the participants was 25.6 ± 3.6 years (range 20–34 years). BMI was 22.9 ± 2.8 kg/m² (range 17.2–32.1 kg/m²) and it was similar to the results of epidemiological studies in Asian men (27, 28). Multiple linear regression analysis was undertaken

with BMD as the dependent variable, and age, height, BMI, amount of calcium intake, smoking and drinking habits, time of exercise and log-transformed leptin levels as the independent variables. It showed a positive association of BMI with BMD at all sites ($\beta = 0.272$, $P = 0.001$ at the lumbar spine; $\beta = 0.211$, $P = 0.005$ at the femoral neck; $\beta = 0.147$, $P = 0.042$ at Ward's triangle; $\beta = 0.189$, $P = 0.017$ at the trochanter; $\beta = 0.234$, $P = 0.003$ at the femoral shaft). Age was independently associated with BMD only at the lumbar spine ($\beta = -0.148$, $P = 0.041$). No associations of BMD with height, calcium intake, smoking, alcohol consumption, time of exercise or serum leptin levels were observed. There was a strong positive correlation between serum leptin levels and BMI ($r = 0.700$, $P < 0.001$).

Genotype frequencies

The genotype distribution and allele frequencies for the polymorphisms of LEPR and ER α are presented in Table 1. All polymorphisms were in Hardy–Weinberg equilibrium. The allele frequencies for the Lys109Arg, Gln223Arg, Ser492Thr, and Pro1019Pro polymorphisms were comparable with those previously reported from Japan (15). The absence of polymorphism at Ala976Asp was also in line with the report from Japan. The allele frequencies for the *Pvu*II and *Xba*I polymorphisms of ER α were not different from those previously shown in Korean women (29). We then analyzed the frequencies of the genotype combinations defined by the *Pvu*II (P) and *Xba*I (X) polymorphisms. The frequencies of the genotype combinations were PPXX, 11 (5.0%); PPXx, 13 (5.9%); PPxx, 12 (5.5%); PpXx, 54 (24.7%); Ppxx, 44 (20.1%); ppXx, 8 (3.7%); and ppxx, 77 (35.2%). The absence of PpXX or ppXX

Table 1 Genotype distribution and allele frequencies of the polymorphisms of the leptin receptor (LEPR) and estrogen receptor α (ER α) genes. Genotype frequencies are expressed as number (%).

Genotype frequencies				Allele frequencies		<i>P</i> value*
LEPR						
Lys109Arg	Lys/Lys 7 (3.2%)	Lys/Arg 61 (27.9%)	Arg/Arg 151 (68.9%)	Lys 0.17	Arg 0.83	0.963
Gln223Arg	Gln/Gln 3 (1.4%)	Gln/Arg 58 (26.5%)	Arg/Arg 158 (72.1%)	Gln 0.15	Arg 0.85	0.662
Ser492Thr	Ser/Ser 218 (99.5%)	Ser/Thr 1 (0.5%)	Thr/Thr 0 (0%)	Ser 0.998	Thr 0.002	0.999
Ala976Asp	Ala/Ala 0 (0%)	Ala/Asp 0 (0%)	Asp/Asp 219 (100%)	Ala 0	Asp 1	—
Pro1019Pro	G/G† 6 (2.8%)	G/A† 37 (16.9%)	A/A† 176 (80.4%)	G† 0.11	A† 0.89	0.085
ER α						
<i>Pvu</i> II	P/P 36 (16.4%)	P/p 98 (44.7%)	p/p 85 (38.8%)	P 0.39	p 0.61	0.693
<i>Xba</i> I	X/X 11 (5.0%)	X/x 75 (34.2%)	x/x 133 (60.7%)	X 0.22	x 0.78	0.995

* Significance is expressed as Hardy–Weinberg equilibrium calculations by χ^2 test. If the *P* value > 0.05 , the genotypes are in Hardy–Weinberg equilibrium.
† G to A transversion at nucleotide 3057 (codon 1019) without amino acid substitution.

genotype was consistent with previous reports (26, 30). The distribution of combination genotypes indicated that the *Pvu*II and *Xba*I polymorphisms were in linkage disequilibrium ($\chi^2 = 104.680$, $P < 0.001$). The PPXX genotype was present five times more frequently, whereas the PpXx and ppXx genotypes showed 1.5-fold higher frequency than expected.

Association of the polymorphism of LEPR gene with BMD

Multiple linear regression analyses were undertaken with BMD as the dependent variable, and with age, BMI and LEPR genotypes as the independent variables. There was no association between BMD and the Lys109Arg polymorphism nor between BMD and the Pro1019Pro polymorphism at either the lumbar spine or proximal femur. The frequencies of genotype variations at the Ser492Thr and Ala976Asp were too low to be analyzed for association with BMD. Because the number of subjects bearing Gln223Gln was too small ($n = 3$), we compared those carrying the Gln223 allele (Gln223Gln or Gln223Arg) with those without (Arg223Arg). No significant differences in age, height, amount of calcium intake, smoking and drinking habits, time of exercise, or serum leptin concentrations were observed between the two groups (Table 2). Body weight and BMI were also not different between the two groups ($P = 0.528$ and $P = 0.307$, respectively). Multiple linear regression analysis revealed that the Gln223Arg polymorphism was independently associated with BMD at the lumbar spine (Table 3). However, this association was not observed at the proximal femur.

The effects of interactions between the LEPR and ER α genotypes on BMD

No significant differences in BMD were noted among the *Pvu*II and *Xba*I genotypes of ER α (data not shown). We also investigated the relationship between the Px haplotype and BMD as previously described (26), but the Px haplotype predicted neither lumbar nor femur neck BMD (data not shown).

LEPR Gln223Arg polymorphism was not in linkage disequilibrium with the ER α genotypes ($\chi^2 = 1.514$, $P = 0.824$ with the *Pvu*II; $\chi^2 = 1.969$, $P = 0.741$ with the *Xba*I genotype). To assess the presence of interactions between LEPR and ER α genotypes, we performed analysis of covariance with the Gln223Arg and *Pvu*II or *Xba*I polymorphisms as the fixed factors. Interactive effects between Gln223Arg and *Pvu*II polymorphisms on BMD were significant ($P = 0.004$) at the lumbar spine, but not at the proximal femur. The effects between Gln223Arg and *Xba*I on BMD were also noted at the lumbar spine and femoral neck ($P = 0.003$ and $P = 0.038$, respectively).

In order to refine the interaction, we determined the difference of BMD in the Gln223Arg genotype in relation to the ER α genotypes. Compared with those without, those subjects with the Gln223 allele had significantly higher BMD at the lumbar spine in the subjects with the PP homozygotes of *Pvu*II (1.362 ± 0.211 g/cm² and 1.190 ± 0.111 g/cm² in the subjects carrying Gln223 allele and those without, respectively, $P = 0.003$, Fig. 1), but not at the proximal femur (data not shown). Associations were also noted at the lumbar spine in the subjects with the X alleles of *Xba*I. (1.305 ± 0.202 g/cm² and 1.184 ± 0.126 g/cm² in the subjects carrying Gln223 allele and those without, respectively, $P = 0.001$). However,

Table 2 Clinical characteristics, serum leptin levels and BMD values according to the Gln223Arg genotype of LEPR in the study subjects. Values are means $\pm$ S.D.

	Gln223Gln Gln223Arg ($n = 61$)	Arg223Arg ($n = 158$)	P value
Age (years)	25.0 $\pm$ 3.3	25.5 $\pm$ 3.8	NS
Height (cm)	174.2 $\pm$ 5.7	173.9 $\pm$ 4.9	NS
Body weight (kg)	68.8 $\pm$ 11.2	69.7 $\pm$ 8.6	NS
BMI (kg/m ²)	22.6 $\pm$ 3.1	23.0 $\pm$ 2.6	NS
Amount of calcium intake (mg/day)	590 $\pm$ 61	592 $\pm$ 77	NS
Amount of smoking (pack-year)	2.03 $\pm$ 3.70	1.63 $\pm$ 3.06	NS
Amount of alcohol intake (g/day)	14.7 $\pm$ 18.1	17.1 $\pm$ 28.1	NS
Time of exercise (hours/day)	1.4 $\pm$ 1.2	1.6 $\pm$ 1.7	NS
Serum leptin concentration (ng/ml)	3.68 $\pm$ 2.02	4.15 $\pm$ 2.39	NS
BMD values (g/cm ²)			
Lumbar spine	1.233 $\pm$ 0.177	1.192 $\pm$ 0.132	NS
Femoral neck	1.081 $\pm$ 0.175	1.047 $\pm$ 0.151	NS
Ward's triangle	1.009 $\pm$ 0.197	0.978 $\pm$ 0.164	NS
Trochanter	0.845 $\pm$ 0.176	0.844 $\pm$ 0.176	NS
Femoral shaft	1.340 $\pm$ 0.179	1.346 $\pm$ 0.173	NS

NS, not significant.

Table 3 Multiple linear regression analysis with BMD as the dependent variable, and age, BMI and the Gln223Arg genotype of LEPR as the independent variables.

BMD site	Age		BMI		Gln223Arg genotype	
	β	P value	β	P value	β	P value
Lumbar spine	-0.165	0.045	0.294	<0.001	-0.137	0.037
	Adjusted R ² = 0.085					
Femoral neck	-0.137	NS	0.257	<0.001	-0.080	NS
	Adjusted R ² = 0.140					
Ward's triangle	-0.158	NS	0.205	0.002	-0.059	NS
	Adjusted R ² = 0.147					
Trochanter	-0.126	NS	0.330	<0.001	-0.003	NS
	Adjusted R ² = 0.116					
Femoral shaft	-0.152	NS	0.267	<0.001	0.024	NS
	Adjusted R ² = 0.102					

the association between the LEPR Gln223Arg polymorphism and lumbar BMD was not significant in those with the Pp heterozygotes or pp homozygotes of *Pvu*II and in those without the X alleles of *Xba*I. No significant differences in age, height, body weight, calcium intake, smoking or alcohol consumption were observed between the two groups at each subgroup analysis (data not shown).

Discussion

To our knowledge, this study is the first report that shows an association between the polymorphism of

the LEPR gene and BMD. Our data indicate that the Gln223Arg polymorphism of LEPR gene is associated with BMD at the lumbar spine in young men, and that this association is dependent on the *Pvu*II and *Xba*I genotypes of ER α . The relative site-specific aspect of the association between the Gln223Arg genotypes and lumbar BMD in comparison with the femur is consistent with the observation of Ducy *et al.* (9). In that study, leptin-deficient ob/ob mice had a markedly increased trabecular bone mass, while no significant change in cortical bone mass was observed, suggesting that the leptin system may have a preferential effect on trabecular bone.

The Gln223Arg polymorphism is within the region encoding the extracellular domain of LEPR and the Gln to Arg substitution has been reported to cause a change in charge (31). Therefore, the Gln223Arg polymorphism might affect the functional characteristics of the receptor. A previous study reported that the leptin-binding affinity was lower in the carriers of the Arg223 allele (32). This supports the hypothesis that the association of the Gln223Arg polymorphism with BMD might be through a change in leptin-binding affinity. However, we cannot exclude the possibility of linkage disequilibrium, considering the fact that there was no difference in serum leptin concentrations among genotypes shown in this and other studies (33).

The Gln223Arg polymorphism has been studied for an association with obesity; some found that the subjects with the Arg allele were more obese than those without (12, 34), but others did not (14, 15). To a certain extent, these results are not surprising considering the polygenic nature of human obesity. We also did

Figure 1 The association with BMD (g/cm²) and the Gln223Arg allele of LEPR at lumbar spine and femoral neck according to the *Pvu*II genotype of ER α . All values are means $\pm$ s.d. The significance is expressed as P values after multiple linear regression analysis with BMD as the dependent variable and with age, BMI and the Gln223Arg genotype of LEPR as the independent variables.

not observe any associations between BMI and LEPR genotypes, even when there were differences in BMD. These findings further suggest that the higher lumbar BMD in those with the Gln223 allele is not simply due to higher BMI itself.

ER α is one of the genes most frequently investigated for the possible association of its polymorphism with BMD in women. Although evidence shows such an association, it has been shown to be dependent on the site of measurement, age, and ethnicity of the study population. Thus, no consensus has been made on the role of ER α polymorphism on female BMD (6). ER α polymorphism may be of interest in men as well in view of the evidence supporting the importance of estrogen on the male skeleton (3), but only a few reports have studied its role in association with male BMD (18, 35). Two studies have reported that ER α polymorphisms are associated with BMD in men (18, 35). In our study, we did not find any association between BMD and the ER α polymorphisms, which might be the result of ethnic differences or the relatively small numbers in the study population.

However, we found that the ER α polymorphisms interact with the LEPR polymorphism to regulate peak bone mass. The association between LEPR Gln223Arg polymorphism and lumbar BMD was significant in the subjects with the PP homozygotes of PvuII or the X allele of XbaI, but not in those with the Pp heterozygotes or pp homozygotes of PvuII and in those without the X alleles of XbaI. These results support the hypothesis that the genotype of ER α may modify the effect of the LEPR gene on bone metabolism. The PvuII and XbaI polymorphisms are approximately 50 bp apart and are in linkage disequilibrium. In our study, the PP homozygotes and X allele had similar influences on the Gln223Arg polymorphism. However, it should be considered that the numbers in our study population were too small to clarify the effects of ER α genotypes on associations between Gln223Arg polymorphism and BMD at all sites, and further study using a larger population is required. Whether and how these intronic polymorphisms of PvuII and XbaI affect ER α function remains to be determined.

In conclusion, this study indicates that the Gln223Arg polymorphism of LEPR is important in the determination of the peak bone mass in men. Furthermore, the association seems to be influenced by ER α genotypes. The variant alleles of the leptin receptor gene could be potential targets for tailored therapeutic interventions for regulation of bone mass in the future.

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