Estrogen receptor α gene polymorphisms (PvuII and XbaI) 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 (ERα) 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 ERα genotype.

Methods: From 219 healthy volunteers aged 20–34 years, we genotyped the Lys109Arg, Gln223Arg, Ser492Thr, Ala976Asp, and Pro1019Pro variants of LEPR and the PvuII and XbaI variants of ERα 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 PvuII and XbaI genotypes of ERα. However, an association between LEPR and BMD was noted in the subjects carrying the PP homozygotes of PvuII or the X alleles of XbaI, 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 ERα 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 (ERα), 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, ERα gene polymorphism has been reported to be associated with bone mineral density (BMD) in boys (18). These results propose that the ERα 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 ERα, 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 ERα genes, and the possible interactive effects of polymorphisms of ERα 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. 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, Aspensaustasse, Reutlingen, Germany) with a sensitivity of 0.1 ng/ml.

Bone mineral densitometry

BMD (g/cm²) of the lumbar spine (L2–L4, antero-posterior) 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 Pro2II and XbaI sites of the ERα 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 ERα 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 HaeIII, MspI, PstI, HaeIII, and HincII (New England Biolabs Inc., Beverly, MA, USA) for the Lys109Arg, Gln223Arg, Ser492Thr, Ala976Asp, Pro1019Pro polymorphisms of the LEPR gene respectively. PvuII and XbaI (New England Biolabs Inc.) were used for the ERα gene. The DNA fragments were then electrophoresed on 3% agarose gels for LEPR and 1% agarose gels for ERα.
**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 \( \chi^2 \) test. Linkage disequilibrium resulting from the non-random association of the genotypes was also assessed by \( \chi^2 \) 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/m\(^2\) (range 17.2–32.1 kg/m\(^2\)) 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\(\alpha\) are presented in Table 1. All polymorphisms were in Hardy–Weinberg equilibrium. The allele frequencies for the Lys109Arg, Gin223Arg, Ser1019Thr, and Pro1019Thr 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 PvuII and XbaI polymorphisms of ER\(\alpha\) were not different from those previously shown in Korean women (29). We then analyzed the frequencies of the genotype combinations defined by the PvuII (\( P \)) and XbaI (\( X \)) 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 \(\alpha\) (ER\(\alpha\)) genes. Genotype frequencies are expressed as number (%).

<table>
<thead>
<tr>
<th>Genotype frequencies</th>
<th>Allele frequencies</th>
<th>( P ) value*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LEPR</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lys109Arg</td>
<td>Lys/Lys</td>
<td>Lys/Arg</td>
</tr>
<tr>
<td>7 (3.2%)</td>
<td>61 (27.9%)</td>
<td>151 (68.9%)</td>
</tr>
<tr>
<td>Gin223Arg</td>
<td>Gin/Gin</td>
<td>Gin/Arg</td>
</tr>
<tr>
<td>3 (1.4%)</td>
<td>58 (26.5%)</td>
<td>158 (72.1%)</td>
</tr>
<tr>
<td>Ser492Thr</td>
<td>Ser/Ser</td>
<td>Ser/Thr</td>
</tr>
<tr>
<td>218 (99.5%)</td>
<td>1 (0.5%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Ala976Asp</td>
<td>Ala/Ala</td>
<td>Ala/Asp</td>
</tr>
<tr>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>219 (100%)</td>
</tr>
<tr>
<td>Pro1019Thr</td>
<td>G/G†</td>
<td>G/A†</td>
</tr>
<tr>
<td>6 (2.8%)</td>
<td>37 (16.9%)</td>
<td>176 (80.4%)</td>
</tr>
</tbody>
</table>

| **ER\(\alpha\)**      |                   |               |
| PvuII                | P/p               | p/p           | P      | p   | 0.693 |
| 36 (16.4%)           | 98 (44.7%)        | 85 (38.8%)    | 0.39    | 0.61 |
| XbaI                 | X/X               | X/x           | X      | x   | 0.995 |
| 11 (5.0%)            | 75 (34.2%)        | 133 (60.7%)   | 0.22    | 0.78 |

*Significance is expressed as Hardy–Weinberg equilibrium calculations by \( \chi^2 \) 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.
The effects of interactions between the LEPR and ERα genotypes on BMD

No significant differences in BMD were noted among the PvuII and XhoI genotypes of ERα (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 ERα genotypes \( \chi^2 = 1.514, P = 0.824 \) with the PvuII: \( \chi^2 = 1.969, P = 0.741 \) with the XhoI genotype). To assess the presence of interactions between LEPR and ERα genotypes, we performed analysis of covariance with the Gln223Arg and PvuII or XhoI polymorphisms as the fixed factors. Interactive effects between Gln223Arg and PvuII polymorphisms on BMD were significant \( (P = 0.004) \) at the lumbar spine, but not at the proximal femur. The effects between Gln223Arg and XhoI 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 ERα genotypes. Compared with those without, those subjects with the Gln223 allele had significantly higher BMD at the lumbar spine with the PP homozygotes of PvuII \( (1.36 \pm 0.211 \text{ g/cm}^2 \) and \( 1.190 \pm 0.111 \text{ g/cm}^2 \) 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 XhoI. \( (1.305 \pm 0.202 \text{ g/cm}^2 \) and \( 1.184 \pm 0.126 \text{ g/cm}^2 \) in the subjects carrying Gln223 allele and those without, respectively, \( P = 0.001 \)). However,
the association between the LEPR Gln223Arg polymorphism and lumbar BMD was not significant in those with the Pp heterozygotes or pp homozygotes of PvuII and in those without the X alleles of XbaI. 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 PvuII and XbaI genotypes of ERα. 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
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.

ERa 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 ERa polymorphism on female BMD (6). ERa 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 ERa polymorphisms are associated with BMD in men (18, 35). In our study, we did not find any association between BMD and the ERa polymorphisms, which might be the result of ethnic differences or the relatively small numbers in the study population.

However, we found that the ERa 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 homozygote of PvuII or the X allele of XbaI, but not in those with the Pp heterozygote or pp homozygote of PvuII and in those without the X allele of XbaI. These results support the hypothesis that the genotype of ERa 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 ERa 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 ERa 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 ERa 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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