의학석사 학위논문

Association between polymorphisms in leptin, its receptor and beta adrenergic receptor genes and bone mineral density in postmenopausal Korean women

한국 폐경 여성에서 leptin, leptin receptor, beta adrenergic receptor 유전자 다형성과 골밀도 사이의 연관성

2013 년 4 월

서울대학교 대학원

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이 희 준

A thesis of the Master’s degree

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지도교수 김 정 구

이 논문을 의학석사 학위논문으로 제출함

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이희준의 의학석사 학위논문을 인준함

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② 본인의 논문을 디지털화하여 인터넷 등 정보통신망을 통한 논문의 일부 또는 전부의 복제.배포 및 전송 시 무료로 제공하는 것에 동의합니다.

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Abstract

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Objective: The purpose of this study was to investigate the association between single nucleotide polymorphisms (SNPs) in the leptin (*LEP*), leptin receptor (*LEPR*), and beta adrenergic receptor (*ADRB*) genes and bone mineral density (BMD) in postmenopausal Korean women.

Methods: The *LEP c.*280G>A, *LEPR c.*326A>G, *c.*668A>G, *c.*1968G>C, *c.*2096C>T, *ADRB2 c.*46A>G, *c.*79C>G, *c.*718T>C, *c.*741G>T, *c.*769G>A, and *ADRB3 c.*190T>C polymorphisms were analyzed in 592 postmenopausal Korean women. Serum levels of leptin, soluble leptin receptor (sLR), osteoprotegerin(OPG), soluble receptor activator of the nuclear factor-κB ligand (sRANKL), bone alkaline phosphatase, and carboxy-terminal telopeptide of type I collagen were measured, and BMDs at the lumbar spine and femoral neck were also examined.

Results: Among polymorphisms measured, the *LEPR c.*1968G>C polymorphism only was found to be related to BMD at the femoral neck, and higher BMD was associated with an increasing number of G alleles (*P*=0.04). Osteoporosis at the femoral neck was 3.27- and 3.89-times more frequently observed in the AG and GG genotypes compared to the AA genotype in the *ADRB2 c.*46A>G polymorphism (*P*=0.024 and 0.015, respectively). However, no significant differences in serum levels of leptin, sLR, free leptin index, OPG, sRANKL, and bone turnover markers were detected among single and haplotype genotypes.

Conclusions: Our results suggest that the *LEPR c.*1968G>C polymorphism may be one of the genetic factors affecting femoral neck BMD in postmenopausal Korean women, and that an analysis of the *ADRB2 c.*46A>G polymorphism may be useful in identifying women at risk of osteoporosis.

Key words: Bone density, Leptin- beta adrenergic receptor, Polymorphism, Bone markers.

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Introduction

Osteoporosis is defined as a skeletal disease characterized by low bone strength, which predisposes a person to an increased risk of fractures.[1] Bone strength is regarded as an integration of bone density and quality. Bone mineral density (BMD) is expressed as grams of mineral per area or volume and accounts for approximately 70% of bone strength.[1-2] We have reported several candidate genetic polymorphisms associated with osteoporosis in Korean women.[3-8] Although osteoporosis is known to be a multifactorial disorder and genetic influence is as high as 70% of individual variance in BMD,[2] genes responsible for the regulation of bone mass and susceptibility to osteoporosis remain to be defined.

In contrast to muscle-derived mechanical effects on bone modeling, peripheral body fat has been reported to influence bone mass via the secretion of endocrine factors.[9] The cytokine-like hormone leptin, which has been widely accepted to be an important regulator of food intake and energy expenditure,[10] appears to stimulate periosteal bone formation through anabolic effects on osteoblasts.[9] Food restriction in mice decreases longitudinal growth and reduces bone mass, but leptin administration restores skeletal growth in mice with low caloric intake and also increases serum osteocalcin, which is a biochemical marker for bone formation.[11] Recombinant leptin treatment also improves markers of bone formation in women with hypothalamic amenorrhea, who have relative leptin deficiency.[12] Interestingly, leptin affects bone centrally through sympathetic nervous system which is an important downstream mediator.[13] Leptin deficiency appears to decrease adrenergic tone[14] and have an effect on the release of noradrenaline from sympathetic nerve fibers.[15] Noradrenaline then binds to beta-2 adrenergic receptors on osteoblasts and inhibits bone formation.

Analyzing genetic variants in leptin (*LEP*) or leptin receptor (*LEPR*) gene is another approach to investigate the association between leptin and bone mass. With regard to the association between *LEPR* polymorphisms and BMD, several studies have been undertaken, however, the results are controversial and dependent on the study population or the type of single nucleotide polymorphism (SNP) evaluated.[16-20] Moreover, the relationship between beta-2 adrenergic receptor (*ADRB2*) gene polymorphisms and bone mass has not been studied thus far. In the present study, we aimed to investigate the relationship between the *LEP*, *LEPR*, *ADRB2*, and *ADRB3* polymorphisms and BMD in postmenopausal Korean women.

Materials and Methods

Study participants

A total of 712 women aged 48 to 65 years were initially included in this study and they attended the Menopause Clinic of Seoul National University Hospital for bone mass examination. All of these women were considered postmenopausal because they had not menstruated for at least one year. Medical history was taken and thorough physical examination was performed. Women with current hepatic disease, renal disease, or diabetes mellitus were excluded after laboratory tests. Women with previous surgical removal of both ovaries were also excluded. Finally, 592 women were enrolled in this study, and no participant was not taking any medication known to affect bone metabolism, such as estrogen, calcium, vitamin D, or calcitonin. All subjects agreed to participate in this study and the study protocol was approved by the Institutional Review Board of Seoul National University Hospital.

Measurements of BMD

BMD was measured at the lumbar spine (L_2-L_4) and femoral neck by dual-energy X-ray absorptiometry (DXA) (DPX-L, Lunar Radiation Corp., Madison, WI). Measurements of BMD were presented in grams per square centimeter, and study participants were classified into three groups based on the World Health Organization criteria.[21] The in vivo coefficient of variation was 1.4% for the lumbar spine and 2.1% for the femoral neck.

Measurements of serum osteoprotegerin (OPG), soluble receptor activator of the nuclear factor-κB ligand (sRANKL), and bone turnover markers

Blood samples were drawn from peripheral veins of all subjects in accordance with the Declaration of Helsinki guidelines. Serum OPG was measured with an enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN). The limit of detection was 0.06 ng/ml, and inter- assay and intra-assay coefficients of variation for OPG were 7.0% and 5.9%, respectively. An enzyme immunoassay kit (Biomedica Gruppe, Wien, Austria) was used to measure serum sRANKL. The minimum detection limit was 1.6 pg/ml, and inter- and intra-assay coefficients of variation for sRANKL were 7.2% and 3.9%, respectively. Serum bone alkaline phosphatase (BAP) levels were determined using an immunoassay kit (Metra Biosystems Inc., Mountain View, CA) with a minimal detection limit of 0.7 U/L and inter- and intra-assay coefficients of variation of 5.2% and 3.9%, respectively. Serum carboxy-terminal

telopeptide of type I collagen (CTX) was measured with a serum CrossLaps One Step enzyme-linked immunosorbent assay kit (Osteometer Biotech, Herlev, Denmark). The limit of detection was 94 pM/L, and intra- and inter-assay coefficients of variation for CTX were 5.4% and 5.0%, respectively.

Measurements of serum leptin and soluble leptin receptor (sLR), and calculation of free leptin index(FLI)

Serum leptin and sLR were measured using an enzyme-linked immunosorbent assay kit (R&D Systems). The minimum detection limit for leptin was 7.8 pg/ml, and inter-assay and intra-assay coefficients of variation were 3.3% and 4.2%, respectively. Serum sLR was measured using an enzyme immunoassay kit (Biomedica Gruppe). The limit of detection for soluble leptin receptor was 0.057 ng/ml, and inter- and intra-assay coefficients of variation were 4.9% and 5.3%, respectively. Free leptin index (FLI) was calculated as the serum leptin divided by sLR (expressed in ng/ng).

Determination of *LEP*, *LEPR*, and *ADRB* polymorphisms

Genomic DNA extraction was performed using a QIAamp DNA blood kit (QIAGEN, Valencia, CA). As shown in Table 1, eleven SNPs in *LEP*, *LEPR*, and *ADRB* were selected based on previous reports[16-20, 22-26] and the SNP database registered by the National Center for Biotechnology Information (dbSNP), which were composed of non-synonymous SNPs or disease-associated SNPs. The call rates ranged from 98% to 100%.

The LEP c.280 G>A, LEPR c.2096C>T, and ADRB2 c.718C>T, c.741G>T, and c.769G>A polymorphisms

These polymorphisms were analyzed by direct DNA sequencing. Briefly, polymorphic regions of genes were amplified by polymerase chain reaction (PCR) using the primers described in Table 1. The PCR products were sequenced using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA) on an automated ABI PRISM 310 DNA sequencer (Applied Biosystems). The direct sequencing of the *c*.718C>T and *c*.741G>T SNPs in *ADRB2* gene were shown in Figure 1.

The LEPR c.668 A>G, and ADRB3 c.190T>C polymorphisms

The polymorphic regions were determined using PCR-restriction enzyme fragment length polymorphism (RFLP) analysis. Genotyping for the polymorphism was performed with the primers shown in Table 1, and the restriction enzymes used for the analyses included *Msp*I for the *LEPR c.*668 A>G polymorphism, and the *BstO*I for the *ADRB3 c.*190T>C polymorphism and then electrophoresed through on agarose gel. A typical gel was shown in Figure 2.

The LEPR c.326A>G, and c.1968G>C and ADRB2 c.46A>G, and c.79C>G polymorphisms

The TaqMan allelic discrimination assay was used to identify these polymorphisms. The TaqMan assay of *c.*326A>G and *c.*1986G>C SNPs in the *LEPR* gene and *c.*46A>G and *c.*79C>G SNPs in the *ADRB2* gene were shown in Figure 3, 4. Polymorphic regions were amplified by PCR with the specific primers shown in Table 1. One allelic probe for each mutant sequence was labeled with a fluorescent amidite matrix-labeled oligonucleotide, and the other contained a fluorescent VIC dye and perfectly matched the wild-type sequence variant. After transfer of the TaqMan assay plates to a Prism 7900HT instrument (Applied Biosystems), the fluorescence intensity in each well of the plate was read and analyzed using automated software (SDS 2.1, Applied Biosystems). For quality control, twenty samples measured by TaqMan assay were also analyzed by direct DNA sequencing. Genotyping errors for the *LEPR c.*326A>G, *c.*1968G>C and *ADRB2 c.*46A>G, and *c.*79C>G polymorphisms were 0.3%, 0.5%. 2.0%, and 0.8%, respectively.

Statistical analyses

All data are expressed as the mean ± standard error and were analyzed using the Statistical Package for Social Sciences 17.0 (SPSS Inc., Chicago, IL). The genotype frequencies for each polymorphism were checked for Hardy-Weinberg equilibrium by the chi-square test or Fisher's exact test. Assessment of linkage disequilibria from non-random associations of alleles was also performed by the chi-square test. Haplotypes of polymorphisms were analyzed using SNP analyzer 1.2A (Istech, Koyang City, Korea). Differences in anthropometric characteristics according to the genotypes were tested by one-way analysis of variance (ANOVA) followed by least significant difference as a post-hoc test. Differences in BMD, serum levels of leptin, FLI, sLR, OPG, sRANKL, and bone turnover markers according to the genotypes were analyzed after adjusting for potential confounders, such as age, years since menopause, and BMI, using an analysis of covariance (ANCOVA). Odd ratios (ORs) and 95% confidence intervals (CIs) for

osteoporosis were estimated using the chi-square test or Fisher's exact test. Significance was accepted for *P* values of <0.05, but after Bonferroni correction in multiple testing, *P* values less than 0.025 were considered significant.

Table 1. PCR primers for SNPs of the leptin (*LEP*), leptin receptor (*LEPR*) and beta adrenergic receptors (*ADRB*) genes

Gene	dbSNP ID	Base (amino acid) Alteration	Primer
LEP	rs17151919	*c.*280G>A(V94M)	F:GTCACCGGTTTGGACTTCAT
			R:AGTGACCTTCAAGGCCTCAG
LEPR	rs1137100	*c.*326A>G(L109A)	F: TGCTTCTTATGTGCAGACAACA
			R:GCTAATGCTTACCTATTTGTTGAAAAACTAAAGA
			VIC:TGAAACAAATGTCTTTCCTTC
			FAM:TGAAACAAATGTCCTTCCTTC
	rs1137101	*c.*668A>G(G223A)	F:ACCCTTTAAGCTGGGTGTCCCAAATAG
			R:AGCTAGCAAATATTTTTGTAAGCAATT
	rs8179183	*c.*1968G>C(L656A)	F: CTATGAGAGGACCTGAATTTTGGAGAA
			R: GATTAATATTAAAATTGGGAATACCTTCCAAAGTAAAGTG
			VIC:ACATTTTTCTCGTTTTTCA
			FAM:ACATTTTTCTCCTTTTTCA
	rs3449590	*c.*2096C>T(T699M)	F:TGCAGGCTGCTTGAAAGATA
			R:AAAGCACTGCAGCCCTTAAA
ADRB2	rs1042713	*c.*46A>G(A16G))	F:CGGCGGCGCCTTCTTG
			R:TGCGTGACGTCGTGGTC
			VIC:CACCCAATAGAAGCCA
			FAM:CACCCAATGGAAGCCA
	rs1042714	*c.*79C>G(Gn27Gu)	F:GCCGGACCACGACGT
			R:TGCCCACCACCCACAC
			VIC:TCGTCCCTTTGCTGCGTG
			FAM: TCGTCCCTTTCCTGCGTG
	rs41320345	*c.*718T>C(P240L)	F:TTCTACGTTCCCCTGGTGAT
	rs41358746	*c.*741G>T(G247H)	R:GTCTTGAGGGCTTTGTGCTC
	rs56100672	*c.*769G>A(G257A)	
ADRB3	rs4994	*c.*190T>C(T64A)	F:CGCCCAATACCGCCAACA
			R: CAACCAGGAGTCCCATCA

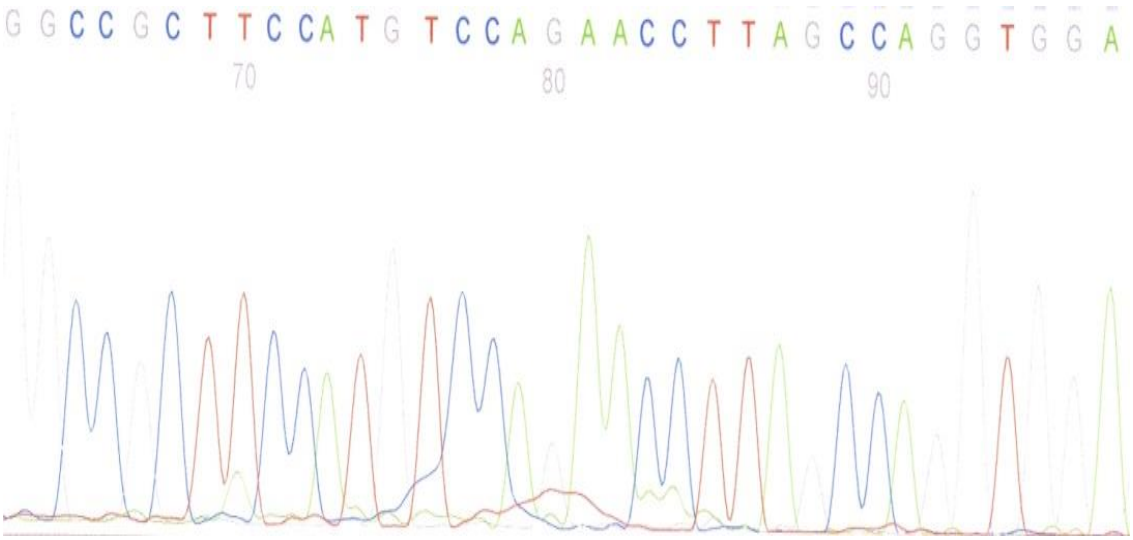

Fig. 1 DNA sequencing of c.718 T>C (A) and c.741 G>T (B) SNPs in beta-2 adrenergic receptor gene

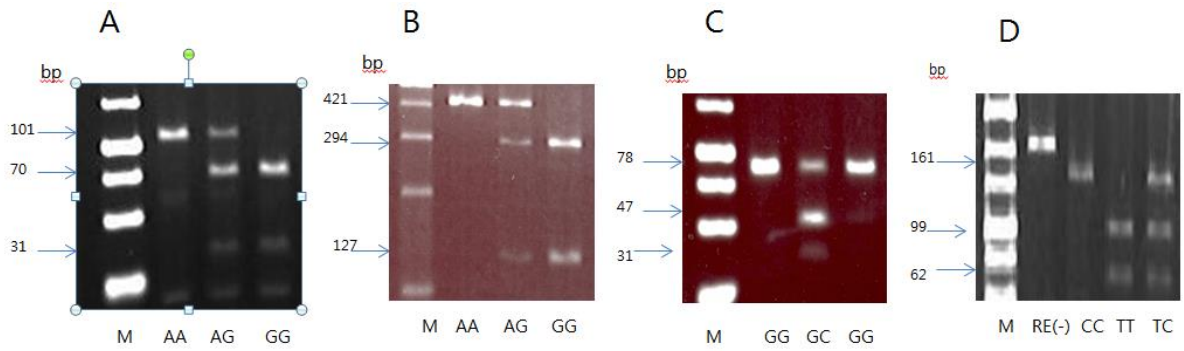

Fig.2. The c.326A>G (HaeIII) SNP (A), c.668A>G (MspI) SNP (B), and c.1968G>C (BstUI) SNP (C) in leptin receptor gene, and c.190T>C(BstOI) SNP (D) in beta-3 adrenergic receptor gene.

M: DNA ladder

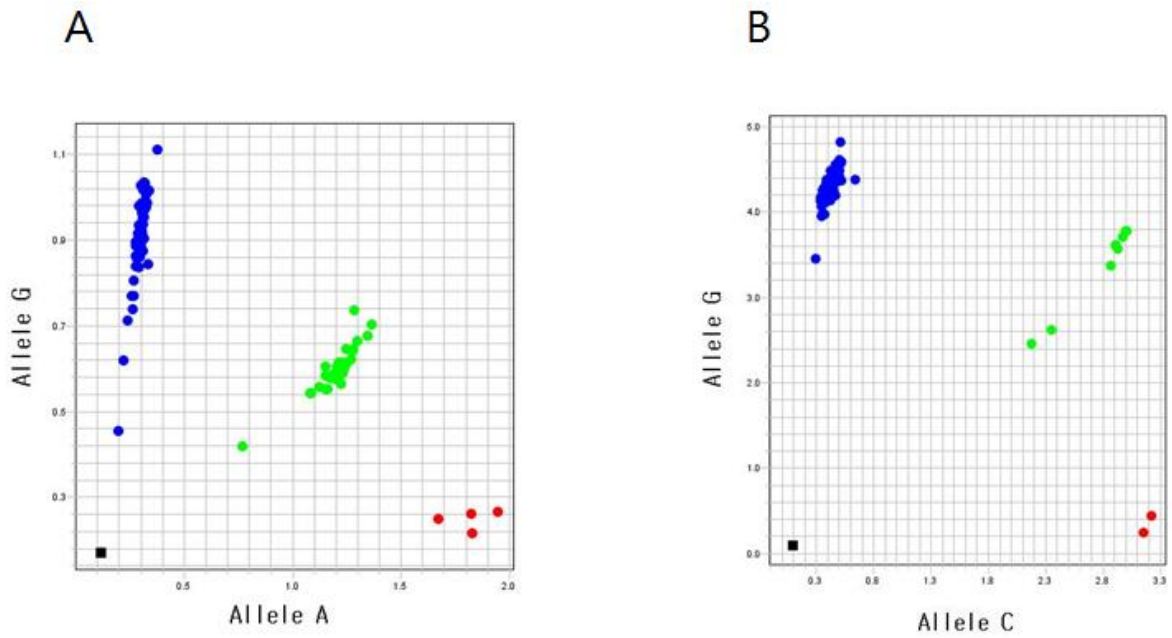

Fig.3. Taqman assay of c.326A>G SNP (A), and c.1968G>C SNP (B) in leptin receptor gene

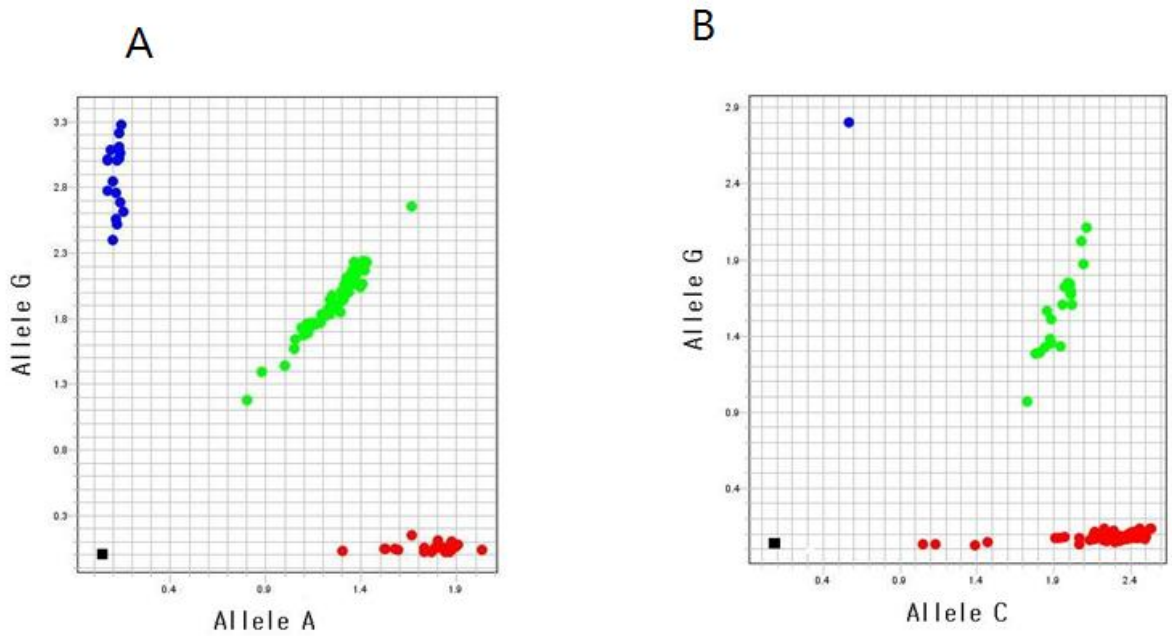

Fig.4. Taqman assay of c.46A>G SNP (A), and c.79C>G SNP (B) in beta-2 adrenergic receptor gene

Results

Single polymorphisms in the *LEP, LEPR,* and *ADRB genes*

The *LEP c.*280G>A, *LEPR c.*2096C>T, and *ADRB2 c.*718T>C, *c.*741G>T, and *c.*769G>A polymorphisms were not observed. Distributions of genotypes in the SNPs of *LEPR* and *ADRB*s polymorphisms were as follows: for *LEPR c.*326A>G, AA 4.2%, AG 27.5%, and GG 68.3%; for *LEPR c.*668A>G, AA 2.2%, AG 34.1%, and GG 63.7%; for *LEPR c.*1968G>C, GG 83.2 %, GC 15.6%, and CC 1.2%; for *ADRB2 c.*46A>G, AA 28.3%, AG 50.3%, and GG 21.4%; for *ADRB2 c.*79C>G, CC 82.5%, CG 17.0%, and GG 0.5%; for *ADRB3 c.*190T>C CC 0.9%, CT 36.1%, and TT 63.1%. These allelic and genotypic frequencies did not deviate from Hardy-Weinberg equilibrium.

No significant differences in age, BMI, or years since menopause were noted according to SNPs in the *LEP*, *LEPR*, and *ADRB* genes. After adjustment for confounding factors, such as age, BMI, and years since menopause, the *LEPR c.*1968G>C polymorphism was the only polymorphism related to BMD at the femoral neck, and an allele dose-effect was observed with BMD at the femoral neck being the highest for the GG genotype, intermediate for CG, and the lowest for the GG (*P*=0.04) (Table 2). Similarly, an allele dose-effect was noted for BMD at femoral neck in the *ADRB2 c.*46A>G polymrophism but did not reach statistical significance. No significant differences in BMD at the lumbar spine and femoral neck were observed according to the ADRB3 c.190T>C, or LEPR c.326 A>G, or c.668A>G polymorphisms (Table 2).

Osteoporosis at the femoral neck were 3.27- and 3.89-fold more frequently observed in the AG and GG genotypes compared to the AA genotype in the ADRB2 c.46A>G polymorphism (P=0.02 and 0.02, respectively) (Table 3). Other LEPR and ADRB polymorphisms were not associated with the prevalence of osteoporosis, and there were no differences in the serum levels of leptin, sLR, FLI, OPG, sRANKL, or bone turnover markers among the genotypes of these polymorphisms (Table 4).

Combined polymorphisms in the *LEPR,* and *ADRB2* genes

In a linkage disequilibrium analysis, significant relationships among *LEPR* polymorphisms were also noted. The combination of these polymorphisms of *LEPR* resulted in 8 haplotype alleles and 20 haplotype genotypes. BMD at the lumbar spine and femoral neck, the risk of osteoporosis, and serum levels of leptin, sLR, FLI, OPG, sRANKL, and bone turnover markers

were not related with the *LEPR* haplotype genotypes (data not shown).

The linkage disequilibrium analysis revealed a significant relationship between the *ADRB2* *c.*46A>G and *c.*79C>G polymorphism. The combined polymorphisms of *ADRB2* gave three haplotype alleles and six different genotypes: AC-GC 39.8%, AC-AC 28.2%, GC-GC 14.5%, AC-GG 10.6%, GC-GG 6.5%, and GG-GG 0.3%. The genotype not carrying the haplotype AC allele and AC heterozygotes showed 3.89- and 3.27-fold higher odds of osteoporosis at the femoral neck compared with AC homozygote respectively (P=0.02 and 0.02, respectively) (data not shown). However, no associations between these *ADRB2* haplotype genotypes and the adjusted serum levels of leptin, sLR, FLI, OPG, sRANKL, and bone turnover markers were noted.

Table 2. Demographic data and bone mineral density in relation to the *LEPR c.*326A>G, *c.*668A>G, 1968G>C, *ADRB2 c.*46A>G, *c.*79C>G and *ADRB3 c.*190T>C genotype in postmenopausal women

Polymorphisms	Genotypes	Age	YSM	BMI	BMD (g/cm^2)	
		(years)	(years)	(kg/m^2)	LS	FN
*LEPR c.*326A>G	AA (n=25)	55.5±1.3	6.4±1.0	24.3±0.5	1.039±0.033	0.865±0.025
	AG (n=162)	57.7±0.5	9.5±0.6	24.3±0.2	0.989±0.013	0.822±0.010
	GG (n=403)	57.5±0.3	9.2±0.4	24.2±0.1	1.003±0.008	0.818±0.006
	P value	0.32	0.16	0.83	0.34	0.20
*c.*668A>G	AA (n=13)	55.9±1.7	6.5±1.4	24.0±0.7	0.985±0.046	0.836±0.035
	AG (n=202)	57.7±0.5	9.2±0.5	24.2±0.2	0.982±0.012	0.814±0.009
	GG (n=377)	57.4±0.3	9.2±0.4	24.3±0.2	1.009±0.009	0.825±0.007
	P value	0.61	0.41	0.90	0.16	0.57
*c.*1968G>C	CC (n=7)	57.4±3.1	9.4±2.4	24.9±1.1	0.984±0.062	0.783±0.048[a,b]
	CG (n=92)	58.3±0.7	9.5±0.7	24.3±0.3	0.976±0.017	0.793±0.013[a,c]
	GG (n=490)	57.3±0.3	9.0±0.3	24.2±0.1	1.006±0.008	0.828±0.006[b,c]
	P value	0.39	0.88	0.80	0.26	**0.04**
*ADRB2 c.*46A>G	AA (n=164)	58.3±0.5	9.9±0.6	24.4±0.2	0.994±0.013	0.835±0.010
	AG (n=292)	57.2±0.4	8.9±0.4	24.2±0.2	1.003±0.010	0.820±0.008
	GG (n=124)	57.1±0.6	9.0±7.6	24.0±0.3	0.992±0.015	0.802±0.012
	P value	0.20	0.35	0.57	0.76	0.10
*c.*79C>G	CC (n=484)	58.6±0.3	9.2±0.3	24.2±0.1	0.996±0.008	0.819±0.006
	CG (n=100)	56.9±7.2	8.9±0.7	24.2±0.3	1.012±0.016	0.826±0.013
	GG (n=3)	61.3±10.0	15.3±8.4	22.6±1.6	0.933±0.095	0.691±0.090
	P value	0.39	0.34	0.63	0.54	0.32
*ADRB3 c.*190T>C	CC (n=5)	55.6±3.6	7.4±1.9	22.6±0.5	0.959±0.074	0.828±0.057
	TC (n=212)	57.3±0.5	9.1±0.5	24.0±0.2	0.999±0.011	0.818±0.009
	TT (n=371)	57.6±0.3	9.3±0.4	24.4±0.2	0.999±0.009	0.822±0.007
	P value	0.70	0.80	0.16	0.87	0.92

LEPR, leptin receptor gene; *ADRB*, beta adrenergic receptor gene; YSM, years since menopause; BMI, body mass index; BMD, bone mineral density; LS, lumbar spine; FN, femur neck
Data are presented as mean ± SE.
P: ANCOVA adjusted for age, years since menopause and BMI
a,b,c: $P<0.05$

Table 3. The odds ratio for osteoporosis in relation to the *LEPR c.*1968G>C, and *ADRB2 c.*46A>G genotypes in postmenopausal women

Polymorphisms	Genotypes	OR (95% CI) for osteoporosis		
		LS	FN	LS and/or FN
*LEPR c.*1968G>C	CC (n=7)	1 (2)*	1 (0)	1 (2)
	CG (n=92)	0.61 (0.11-3.39) (18)	Unavailable (8)	0.69 (0.13-3.85) (20)
	GG (n=490)	0.46 (0.09-2.42) (76)	Unavailable (26)	0.48 (0.09-2.53) (79)
	P value	0.32	0.43	0.24
	Non-GG (n=99)	1.38 (0.79-2.38) (20)	1.56 (0.69-3.57) (8)	1.48 (0.87-2.53) (22)
	GG (n=490)	1 (76)	1 (26)	1 (79)
	P value	0.25	0.28	0.14
*ADRB2 c.*46A>G	AA (n=164)	1 (24)	1 (4)	1 (25)
	AG (n=292)	1.18 (0.70-2.01) (49)	**3.27 (1.11-9.66)** (22)**	1.18 (0.70-1.99) (51)
	GG (n=124)	1.47 (0.80-2.73) (25)	**3.89 (1.21-12.54)** (11)**	1.55 (0.85-2.83) (27)
	P value	0.46	**0.045**	0.35
	AA	1 (24)	1 (4)	1 (25)
	Non-AA	1.27 (0.77-2.09) (74)	**3.46 (1.20-9.91)** (33)**	1.29 (0.79-2.11) (78)
	P value	0.39	**0.02**	0.31

LEPR, leptin receptor gene; *ADRB*, beta adrenergic receptor gene; OR, odds ratio; CI, confidence interval; LS, lumbar spine; FN, femur neck

Data are presented as odds ratio (95% confidence interval).

P: chi-square test

*: number of women with osteoporosis

** *P* value less than 0.025 are considered significant after Bonferroni's correction

Table 4. Serum levels of bone markers, leptin, sLR, free leptin index, OPG and sRANKL in relation to the *ADRB2 c.*46A>G, and *c.*79C>G genotypes in postmenopausal women

Polymorphisms	Genotypes	BAP (U/L)	CTX (pM/L)	Leptin (ng/ml)	sLR (ng/ml)	FLI (ng/ng)	OPG (ng/ml)	sRANKL (pg/ml)	$sRANKL^{x1000}$/OPG
*ADRB2 c.*46A>G	AA (n=89)	17.5±0.8	1524.7±424.9	9.9±0.9	33.5±1.7	0.34±0.03	8903.5±365.9	6.6±0.9	0.8±0.1
	AG (n=148)	17.4±0.6	2077.5±326.8	9.4±0.6	35.4±1.1	0.30±0.02	9204.6±250.6	8.0±0.6	0.9±0.1
	GG (n=59)	16.3±1.0	1571.5±518.9	8.4±1.1	35.2±2.3	0.27±0.05	9354.8±442.7	6.5±0.9	0.8±0.1
	P value	0.60	0.51	0.60	0.65	0.45	0.71	0.25	0.36
*c.*79C>G	CC (n=250)	17.4±0.5	1797.0±294.6	9.5±0.5	35.4±1.0	0.30±0.02	9243.4±201.7	7.4±0.5	0.9±0.1
	Non-CC (n=51)	16.3±1.0	1807.0±560.9	8.6±0.9	33.5±1.9	0.30±0.04	8735.0±420.6	8.4±1.1	1.0±0.1
	P value	0.34	0.99	0.37	0.37	0.98	0.28	0.43	0.26

ADRB, beta adrenergic receptor gene; BAP, bone alkaline phosphatase; CTX, CrossLaps; sLR, soluble leptin receptor; FLI, free leptin index; OPG, osteoprotegerin; sRANKL, soluble receptor activator for nuclear factor-kappa B

Data are presented as mean ± SE.

P: ANCOVA adjusted for age, years since menopause and BMI

Discussion

Body weight has been known to impact bone density and bone turnover,[27] and a meta-analysis based on a number of prospective studies revealed that lower BMI might confer a risk of future fracture.[28] Several mechanisms for an obesity-bone association have been suggested: body mass effect on load bearing on the skeleton, hormonal factors such as estrogen, and the secretion of factors associated with adipose tissue.[27] Since osteoblasts and adipocytes share common progenitor cells in the bone marrow,[29] the association between leptin and bone mineral density seems to be quite plausible. It has been known that leptin stimulates bone formation directly through anabolic effects on osteoblasts and also affects ADRB, which increases trabecular bone remodeling.[9] In the search for bone regulating genes in Korean women, the relationships between BMD and eleven SNPs in the *LEP-LEPR-ADRB* genes were analyzed in the present study, and associations between bone status and the *LEPR c.*1968G>C and *ADRB2 c.*46A>G polymorphisms were found.

The human *LEP* gene has been mapped to chromosome 7q31.3 and is composed of three exons and two introns, spanning approximately 18 kb. Leptin acts through the LEPR, which is a transmembrane domain receptor, and the *LEPR* gene is located on chromosome 1p31. We previously reported that no association existed between the *c.*326A>G and *c.*668A>G polymorphisms in *LEPR* and BMD in postmenopausal Korean women,[18] but the number of participants in the previous study was very small (n=118) and possible selection bias might have been included. Because the study population was recruited at the health promotion center of the university hospital, the participants would have higher socioeconomic status and would be more interested in their health or would be healthier than general population. Therefore, to extend the previous study, we analyzed these polymorphisms in larger women in the present study (n=592) and confirmed the previous findings. These findings are in agreement with the results of other studies reporting no association between BMD and the *LEPR c.*668A>G polymorphism in Belgian men aged older than 70 years[16] and in healthy Caucasian prepubertal boys.[20] In contrast to these results, significantly lower BMD at the femoral neck was reported in AG heterozygotes of the *c.*668A>G polymorphism in the Danish population.[17] Koh et al.[19] have also shown that the *c.*668A>G polymorphism was associated with BMD at lumbar spine. However, the study population included relatively young males aged 20 to 34 years in whom peak bone mass would be reached, considering their age. Therefore, the results cannot be extrapolated to postmenopausal women in whom the lifetime risk of osteoporotic fracture is higher compared to

males.

In the present study, we found that the *LEPR c.*1968G>C polymorphism was related to BMD at the femoral neck, and higher BMD was observed with an increasing number of G alleles. By contrast, Fairbrother et al.[17] did not find any significant difference in BMD according to the *c.*1968G>C polymorphism in postmenopausal Danish women. This discrepancy may be due to the differences in study participants, including ethnicity. Their study subjects were older (approximately 71 years) than those (approximately 57 years) in the present study. The frequency of the C allele is also significantly different between the Danish population (16.0%) and our study participants (9.0%) (*P*=0.02). The mechanism by which the *LEPR c.*1968G>C polymorphism may affect BMD remains to be elucidated. This polymorphism leads to an amino acid substitution from leucine to alanine and is predicted to cause a possible damaging effect in the activity of the protein according to the Polyphen-2 online database (http://genetics.bwh.harvard.edu/pph2), which demonstrates a predicted effect of human non-synonymous SNPs. However, considering that the *LEPR c.*1968G>C polymorphism was not associated with serum sLR level in the present study, as the *LEPR c.*326A>G and *c.*668A>G polymorphisms were in other studies,[30-31] the possibility that this *LEPR c.*1968G>C polymorphism may be in linkage disequilibrium with other functional polymorphisms[31] of *LEPR*, which are associated with BMD or the level of serum sLR, cannot be excluded.

Studies evaluating the relationship between obesity and LEPR SNPs have been inconclusive. Some investigators have identified a positive association,[32-33] whereas others have not.[34-35] In the present study, no significant differences in BMI were noted according to the *LEPR* SNPs. However, considering a polygenic trait of obesity, this discrepancy is not a surprising finding.

Beta-adrenergic receptors have been detected on osteoblast and osteoclast,[36] and adrenergic stimulation promotes bone resorption.[37-38] The *ADRB2* gene is located on chromosome 5q31-q32 and is composed of a single exon of 2015 nucleotides.[39] The frequency of the G allele of the *ADRB2 c.*46A>G polymorphism in the present study participants was comparable to that of other studies performed in the Korean population[40-41] BMD at the femoral neck was found to decrease with an increase of one copy of the G allele of *ADRB2 c.*46A>G polymorphism. Furthermore, the odds for osteoporosis at femoral neck were significantly higher in the AG and GG genotypes of the *ADRB2 c.*46A>G polymorphism compared to the AA genotype. In other words, women with the AG or GG genotypes have a higher risk of osteoporosis compared to those with the AA genotype. To the best of our knowledge, this is the first report showing the

relationship between the *ADRB2 c.*46A>G polymorphism and osteoporosis phenotype.

With regard to the *ADRB3 c.*190T>C polymorphism, no differences in BMD were noted according to genotype in the present study. This finding is in agreement with the results of investigators reporting no association between the *ADRB3 c.*190T>C polymorphism and BMD at the lumbar spine and femoral neck in Japanese girls[24] and Caucasian elderly women.[26] Ogawa et al. reported no difference in BMD at the lumbar spine but did identify differences in total body BMD according to the genotypic frequencies of the *c.*190T>C polymorphism in postmenopausal Japanese women,42 however, they compared the Z score between groups (CC+TC vs TT) in contrast to the present study.

There are some limitations to the present study. First, limited statistical power may have been resulted from the relatively small number of study participants. Therefore, the significant results in our study should be interpreted carefully. Second, other potential confounders, such as physical activity and smoking, were not assessed. Finally, only a limited number of polymorphisms in the LEP, LEPR, and ADRB genes were analyzed. Further large-scale studies including participants representing the general population or other ethnic groups are necessary.

Conclusions

The *LEPR c.*1968G>C polymorphism was found to be related to BMD at the femoral neck, and an allele dose-effect was noted with G allele tending toward higher BMD. Osteoporosis at the femoral neck was 3.27- and 3.89-fold more frequently observed in the AG and GG genotypes of the *ADRB2 c.*46A>G polymorphism compared to the AA genotype. Therefore, the *LEPR c.*1968G>C polymorphism may be one of the genetic factors affecting femoral neck BMD in postmenopausal Korean women, and evaluating the *ADRB2 c.*46A>G polymorphism may be useful for identifying Korean women at risk of osteoporosis.

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국문 초록

서론: 본 연구에서는 폐경 후의 한국 여성에서 leptin (*LEP*), leptin receptor (*LEPR*), beta adrenergic receptor (*ADRB*) 유전자 다형성과 골밀도의 연관성을 규명하고자 하였다.

방법: 폐경 후의 한국 여성 592 명에서 *LEP c.*280G>A, *LEPR c.*326A>G, *c.*668A>G, *c.*1968G>C, *c.*2096C>T, *ADRB2 c.*46A>G, *c.*79C>G, *c.*718T>C, *c.*741G>T, *c.*769G>A, and *ADRB3 c.*190T>C 다형성을 조사하였다. 그리고, 혈청내의 leptin, soluble leptin receptor (sLR), osteoprotegerin(OPG), soluble receptor activator of the nuclear factor-κB ligand (sRANKL), bone alkaline phosphatase, and carboxy-terminal telopeptide of type I collagen 을 측정하였고, 요추와 대퇴골경부의 골밀도를 측정하였다.

결과: 유전자의 다형성 중에서, *LEPR c.*1968G>C 다형성만이 대퇴골경부의 골밀도와 연관성이 있었다. 그리고, 증가된 골밀도는 G 대립유전자의 증가와 관련이 있었다 (*P*=0.04). 또한 대퇴골경부의 골밀도는, ADRB2 c.46A>G 다형성에서 AA 유전자형에 비해 AG, GG 유전자형에서 각각 3.27 배, 3.89 배 높게 관찰되었다 (*P*=0.024, *P*=0.015). 그러나, 혈청내의 leptin, sLR, free leptin index, OPG, sRANKL, 골교체인자는 유전자형 간에 차이가 없었다.

결론: *LEPR c.*1968G>C 다형성은 폐경 후 한국 여성에서 대퇴골경부 골밀도에 영향을 미치는 유전 인자 중 하나일 가능성이 있다. 그리고, *ADRB2 c.*46A>G 다형성 분석을 통해 골다공증의 위험도가 높은 폐경 후 여성을 선별하는데 유용할 수 있다.

주요어: 골밀도, Leptin, Beta adrenergic receptor, 다형성, 골표지인자

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