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Full-length article

Association of polymorphisms in low-density lipoprotein receptor-related protein 5 gene with bone mineral density in postmenopausal Chinese women[1]

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Key words

bone density; low-density lipoprotein receptor-related protein 5; polymorphism

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Abstract

Aim: To investigate the possible association of Q89R, N740N and A1330V polymorphisms in low-density lipoprotein receptor-related protein 5 (LRP5) gene with bone mineral density (BMD) in postmenopausal Chinese women. **Methods:** Q89R, N740N and A1330V genotypes were determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) in 647 unrelated healthy postmenopausal Han Chinese women aged 43–76 years in Shanghai. BMD at lumbar spine 1–4 and the left proximal femur including the femoral neck, trochanter and Ward's triangle were measured by dual-energy X-ray absorptionmetry in all subjects. **Results:** The distribution of the Q89R, N740N and A1330V genotypes in this population was as follows: QQ 80.5%, QR 18.7%, and RR 0.8%; TT 66.9%, TC 31.1%, and CC 2.0%; AA 68.0%, AV 29.7%, and VV 2.3%. The frequencies of the Q89R, N740N and A1330V genotypes and alleles did not deviate from the Hardy-Weinberg equilibrium. We found that the Q89R and A1330V polymorphisms were in linkage disequilibrium in our population (χ^2=13.50, P<0.01). Both before and after adjusting for age, years since menopause, height, and weight, the Q89R or N740N genotypes were significantly associated with BMD at the femoral neck (P<0.05). Subjects with the Q89R QQ genotype or the N740N TT genotype had a significantly higher BMD at the femoral neck, compared with those with the QR/RR or TC/CC genotypes, respectively. No significant association was found between A1330V polymorphism and BMD at any site. **Conclusion:** Our findings suggest that the LRP5 gene is a candidate for the genetic determination of BMD in postmenopausal Chinese women.

Introduction

Osteoporosis is characterized by a decrease in bone mass as well as a deterioration of the bone architecture, resulting in an increased risk of fracture. The disease is multifactorial, and it depends on environmental and genetic factors. Twin studies have shown that genetic factors account for 60%–80% of the variance in bone mineral density (BMD)[1–3], which is the best predictor of the risk of osteoporosis. Several candidate genes that may contribute to BMD have been identified. Vitamin D receptor (VDR), estrogen receptor-alpha (ER-alpha), and collagen type I alpha 1 (COL1A1) genes are three important candidate genes that could potentially regulate BMD. Association and linkage studies have been performed in order to identify these candidate genes in the pathogenesis of osteoporosis[4–8]. However, their effect on the variation of BMD in the general population is controversial[9–11].

Recently, osteoporosis-pseudoglioma (OPPG), an autosomal recessive disease characterized by low bone mass, childhood fractures and abnormal eye development, has been shown to be due to an inherited loss of function of the gene for low-density lipoprotein receptor related protein 5 (LRP5)[12]. Moreover, two independent studies have sug-

gested that a mutation (G171V) in the LRP5 gene is associated with high bone mass (HBM)[13,14]. In addition to the above mentioned G171V mutation in the LRP5 gene, Gong *et al*[12] identified three potential disease-associated missense mutations in regions encoding the LRP5 extracellular domain: R494Q, R570W and V667M. More recently, mutation analysis have identified seven novel sequence variants in the human LRP5 gene[15]. Two of them are missense mutations (c.314A>G: Q89R and c.4037C>T: A1330V). Although Koller *et al*[16] found the evidence that a quantitative trait locus (QTL) contributed to normal variation in BMD on chromosome 11q12–13 (the chromosome region where LRP5 is located), only a few studies have investigated the association between LRP5 gene polymorphisms and variation in BMD in the general population[17–20]. However, these studies yielded inconsistent reports of the association between candidate loci and BMD, and only small groups of subjects were studied in the Japanese and Korean studies. In the present study, we investigated the association between BMD and Q89R, A1330V, and N740N polymorphisms in the LRP5 gene in 647 postmenopausal Chinese women.

Materials and methods

Study population The study population comprised of 647 unrelated, postmenopausal, healthy volunteers aged 43–76 years (mean±SD, 60.1±6.3 years) living in Shanghai, China. All participants were of the Han ethnic group. The clinical data taken included questions on medical history, including medication, and a survey of the incidence of disease. A physical checkup was carried out on all subjects, and all were found to be in good health. No participant had medical complications or was undergoing treatment for conditions known to affect bone metabolism, such as hyperthyroidism, diabetes mellitus, primary hyperparathyroidism, renal failure, pituitary and adrenal disease, or rheumatic disease. Postmenopausal women who had experienced early menopause (before 40 years of age) and those who had undergone ovariectomy or who were receiving estrogen replacement therapy were excluded. The study protocol was approved by the Committee on the Ethics of Human Research of Shanghai Jiaotong University Affiliated Sixth People's Hospital.

BMD measurements A total of 647 subjects were measured for BMD. The BMD of the lumbar spine 1–4 (L1–4) and the left proximal femur including femoral neck, trochanter, and Ward's triangle was measured by dual-energy X-ray aborptionmetry (DXA) on a Hologic QDR 2000 (Hologic, Bedford, MA, USA). The machine was calibrated daily, and the coefficient of variation (CV) values of the DXA measurements (which were obtained from 7 individuals repeatedly measured 5 times) at L1–4, the femoral neck, trochanter, and Ward's triangle were 0.9%, 1.93%, 1.48% and 2.85%, respectively[6,21]. The long-term reproducibility of our DXA data during the trial based on weekly repeated phantom measurements was 0.45%.

Genotyping DNA was isolated from peripheral blood leukocytes using conventional methods. Using the methods described by Okubo *et al*[15], we genotyped subjects with the LRP5 Q89R, N780N and A1330V polymorphisms. Genomic DNA (0.1 μg) was carried out in 30 μL buffer solution (10 mmol/L Tris-HCL, 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 200 μmol/L of each of the four deoxyribonucleotides [dNTPs], 2.5 U of *Tag* polymerase, and 0.25 μmol/L of each primer).

Table 1. Three LRP5 gene polymorphisms were identified in this study.

Location	Sequence alteration	Amino acid change	PCR primer for RFLP detection	Restriction enzyme	Fragment size /bp
Exon 2	c.314 A>G	Q89R	Forward: 5'-TCTGGGCATAGTGCTCCATC-3' Reverse: 5'-TTCCGGGATGTGCCATTGAG-3'	*Ava*II	Wild (Q): 436 Variant (R): 274+162
Exon 10	c.2268 T>C	N740N	Forward: 5'-CTACTGGGCCGACACTGGGATTAA-3' Reverse: 5'-ACAGCTCTAATCACTGAGGG-3'	*Ase*I	Wild (t): 216+21 Variant (c): 237
Exon 18	c.4037 C>T	A1330V	Forward: 5'-GACTGTCAGGACCGCTCACACG-3' Reverse: 5'-AAGGTTTTCAGAGCCCCTAC-3'	*Dra*III	Wild (A): 143 Variant (V): 119+24

The polymerase chain reaction (PCR) was performed by using the following steps: 94 ºC for 5 min and then 94 ºC for 1 min, 56 ºC for 1 min, 72 ºC for 1 min, for 30 cycles, and 72 ºC for 7 min. The PCR primers and restriction endonucleases used for genotyping are summarized in Table 1. The PCR products were digested with *AvaI* I, *Ase* I, and *Dra*III restriction endonucleases, respectively. The Q89R genotypes were separated by electrophoresis in 1.5% agarose gel. The QQ genotype produces a 436 bp fragment, the RR genotype produces 274 bp and 162 bp fragments, and the heterozygous QR genotype produces 436 bp, 274 bp and 162 bp fragments. The N740N and A1330V genotypes were separated by electrophoresis on 8.0% polyacrylamide gel electrophoresis gels. The N740N CC genotype produces a 237 bp fragment, the TT genotype produces 216 bp and 21 bp fragments, and heterozygous TC genotypes produce 237 bp, 216 bp and 21 bp fragments. The A1330V AA genotype produces a 143 bp fragment, the VV genotype produces 119 bp and 24 bp fragments, and the heterozygous AV genotype produces 143 bp, 119 bp and 24 bp fragments.

Statistical analysis Allele frequencies were estimated by using the gene counting method, and the chi-squared test was used to identify significant departures from the Hardy-Weinberg equilibrium and linkage disequilibrium between genotypes. The relationship between various LRP5 genotypes and BMD was analyzed by using the unpaired Student's *t*-test. All associations were further evaluated using multiple linear regression analysis to adjust for risk factor. BMD was a dependent variable and the independent variables included age, height, weight, years since menopause, and LRP5 Q89R genotype (0=QQ, 1=QR+RR), N740N genotype (0=TT, 1=TC+CC), and A1330V genotype (0=AA, 1=AV+VV). $P<0.05$ was considered to be statistically significant. All statistical calculations were performed using the SPSS 9.0 program (SPSS, Chicago, IL, USA).

Results

Frequency distribution of LRP5 gene polymorphisms The genotype distribution and allele frequencies of polymorphisms of the LRP5 gene are shown in Table 2. Frequencies of the Q89R, N740N, and A1330V genotypes and alleles did not deviate from Hardy-Weinberg equilibrium. Strong linkage disequilibrium was found between Q89R and A1330V polymorphisms in our population ($\chi^2=13.50$, $P<0.01$; Table 3). However, no linkage disequilibrium was found between Q89R and N740N or between A1330V and N740N polymorphisms in these subjects.

Association between BMD and LRP5 genotype Because the frequencies of the Q89R RR, N780N CC and A1330V VV genotypes were all very low, we compared the background parameters and BMD in the QQ and QR/RR, TT and TC/CC, and AA and AV/VV groups for further analysis. The association between BMD and LRP5 genotype was analyzed us-

Table 2. Genotype distribution and allele frequencies of polymorphisms of the LRP5 gene in 647 postmenopausal Chinese women.

Polymorphism	Genotype frequencies			Allele frequencies	
Q89R	QQ	QR	RR	Q	R
	521 (80.5%)	121 (18.7%)	5 (0.8%)	0.899	0.101
N740N	T T	TC	CC	T	C
	433 (66.9%)	201 (31.1%)	13 (2.0%)	0.825	0.175
A1330V	AA	AV	VV	A	V
	440 (68.0%)	192 (29.7%)	15 (2.3%)	0.828	0.172

Table 3. Frequency of LRP5 genotypes by combination of Q89R and A1330V polymorphisms. Values represent the observed number of subjects with the combined genotypes of Q89R and A1330V; in parentheses are the expected numbers under linkage equilibrium. $\chi^2=13.50$; $P<0.01$ for linkage disequilibrium.

		A1330V polymorphic site		
		AA	AV	VV
Q89R	QQ	389 (355.61)	124 (155.17)	8 (12.13)
polymorphic	QR	48 (79.90)	66 (34.87)	7 (2.72)
site	RR	3 (4.49)	2 (1.96)	0 (0.15)

ing the unpaired Student's *t*-test (Table 4). BMD at the femoral neck was significantly higher in subjects with the Q89R QQ genotype than in the combined group with QR/RR genotypes ($P<0.05$). A similar finding was observed for the N740N genotype; that is, that subjects with the N740N TT genotype had significantly higher BMD at the femoral neck compared with those with TC/CC genotypes ($P<0.05$). Moreover, we further used multiple linear analysis to adjust for age, years since menopause, height, and weight. We found that BMD at the femoral neck was significantly associated with the Q89R polymorphism and the N740N polymorphism ($P<0.05$; Tables 5, 6). However, no significant association was found between the A1330V genotype and BMD at any site according to the unpaired Student's *t*-test or multiple linear analysis (Tables 4, 7).

Discussion

In this cross-sectional study of postmenopausal Chinese women, we found that differences existed in the frequencies of the Q89R, N740N and A1330V LRP5 polymorphisms compared with frequencies in Caucasian people; in particular,

Table 4. BMD and other characteristics according to Q89R, N740N and A1330V polymorphisms in the LRP5 gene in postmenopausal Chinese women. Mean±SD. $^cP<0.05$ *vs* QR/RR genotypes. $^eP<0.05$ *vs* TC/CC genotypes.

	Q89R genotype		N740N genotype		A1330V genotype	
	QQ	QR/RR	TT	TC/CC	AA	AV/VV
Number	521	121/5	433	201/13	440	192/15
Age (years)	59.8±6.2	60.2±6.8	59.9±6.3	59.8±6.2	60.0±6.3	59.5±6.2
Years since menopause (years)	11.3±8.5	11.7±8.1	11.3±8.9	11.4±7.4	11.7±9.0	10.4±6.5
Height (cm)	154.5±5.4	154.3±5.9	154.8±5.3	153.7±5.8	154.7±5.2	153.8±5.9
Weight (kg)	58.5±8.3	59.0±9.4	58.9±8.6	57.9±8.3	59.2±8.6	57.4±8.1
L1–4 BMD (g/cm^2)	0.798±0.140	0.795±0.146	0.805±0.142	0.782±0.138	0.803±0.140	0.786±0.143
Femoral neck BMD (g/cm^2)	0.662±0.114^c	0.644±0.108	0.665±0.117^e	0.645±0.103	0.664±0.115	0.646±0.109
Trochanter BMD (g/cm^2)	0.534±0.100	0.523±0.099	0.535±0.103	0.525±0.092	0.535±0.101	0.525±0.096
Ward's triangle BMD (g/cm^2)	0.493±0.144	0.469±0.135	0.494±0.149	0.477±0.128	0.494±0.144	0.476±0.143

Table 5. Multiple linear analysis with BMD as the dependent variable, and age, years since menopause, height, weight and Q89R genotype as the independent variables.

BMD site	Age	Years since menopause	Height	Weight	Q89R	
	β(*P*)	β(*P*)	β(*P*)	β(*P*)	β(*P*)	R^2
L1–4	-0.285 (0.000)	0.028 (0.640)	0.012 (0.819)	0.463 (0.000)	-0.017 (0.689)	0.291
Femoral neck	-0.436 (0.000)	0.069 (0.163)	-0.054 (0.151)	0.502 (0.000)	-0.065 (0.039)	0.379
Trochanter	-0.335 (0.000)	-0.029 (0.977)	-0.033 (0.397)	0.482 (0.000)	-0.042 (0.194)	0.334
Ward's triangle	-0.438 (0.000)	0.015 (0.774)	-0.025 (0.525)	0.334 (0.000)	-0.064 (0.056)	0.289

Table 6. Multiple linear analysis with BMD as the dependent variable, and age, years since menopause, height, weight and N740N genotype as the independent variables.

BMD site	Age	Years since menopause	Height	Weight	N740N	
	β(*P*)	β(*P*)	β(*P*)	β(*P*)	β(*P*)	R^2
L1–4	-0.283 (0.000)	0.026 (0.672)	0.008 (0.874)	0.462 (0.000)	-0.032 (0.462)	0.291
Femoral neck	-0.436 (0.000)	0.063 (0.197)	-0.058 (0.118)	0.499 (0.000)	-0.067 (0.033)	0.379
Trochanter	-0.335 (0.000)	-0.004 (0.938)	-0.034 (0.374)	0.481 (0.000)	-0.028 (0.384)	0.333
Ward's triangle	-0.438 (0.000)	0.011 (0.831)	-0.028 (0.483)	0.331 (0.000)	-0.044 (0.190)	0.287

Table 7. Multiple linear analysis with BMD as the dependent variable, and age, years since menopause, height, weight and A1330V genotype as the independent variables.

BMD site	Age	Years since menopause	Height	Weight	A1330V	
	β(*P*)	β(*P*)	β(*P*)	β(*P*)	β(*P*)	R^2
L1–4	-0.286 (0.000)	0.027 (0.658)	0.010 (0.851)	0.462 (0.000)	-0.017 (0.695)	0.291
Femoral neck	-0.437 (0.000)	0.064 (0.193)	-0.055 (0.142)	0.497 (0.000)	-0.044 (0.166)	0.376
Trochanter	-0.335 (0.000)	-0.003 (0.949)	-0.033 (0.401)	0.480 (0.000)	-0.013 (0.702)	0.332
Ward's triangle	-0.439 (0.000)	0.011 (0.841)	-0.027 (0.502)	0.329 (0.000)	-0.045 (0.186)	0.287

the Q89R polymorphism is very rare in Caucasian people[22,23]. However, the frequencies of genotypes and alleles of Q89R, N740N and A1330V LRP5 polymorphisms in Chinese women are similar to those found in Japanese and Korean subjects[15,17]. We found that the prevalent frequencies of the Q, T and A alleles in Chinese women were 89.9%, 82.5%, and 82.8%, respectively, as compared with 92%, 81%, and 82%, respectively, in Japanese subjects; furthermore, the frequencies of the Q89R Q and A1330V A alleles were 92% and 85% in Korean subjects. Therefore, LRP5, similar to other candidate genes (eg, VDR and COL1A1) had significantly different frequencies of genotypes and alleles in various ethnic groups[9,24].

In the present study, a significant association was observed between the Q89R genotype or the N740N genotype and BMD at the femoral neck both before and after adjusting for confounding factors, and subjects with the QQ genotype or the TT genotype had significantly higher BMD. Our findings are consistant with those of Koh *et al*[17], who recently reported that the Q89R polymorphism was significantly associated with BMD at the femoral neck and Ward's triangle in 219 young Korean men. After adjusting for age, weight, and height, a marginal association was observed at the femoral neck (P=0.098). However, the N740N polymorphism was not investigated in Korean men. The Q89R and A1330V polymorphisms are located in exon 2 and exon 18 in the LRP5 gene, respectively. The Q89R and A1330V polymorphisms were in linkage disequilibrium in our study population. This finding is similar to that for a Korean population[17], but differs from that for a European population[22]. Although the A1330V polymorphism is a functional mutation, no significant association was observed between the A1330V polymorphism and BMD either in postmenopausal Chinese women or in young Korean men. Similarly, Ferrari *et al*[18] failed to find a significant association between c.4037C>T (A1330V) polymorphism and lumbar bone mineral content and bone area in 889 healthy Caucasian people of both sexes.

LRP5 is a single pass membrane receptor whose extracellular domain contains four modules consisting of six YWTD repeats followed by an epidermal growth factor (EGF)-like motif and an LDLR-like ligand-binding domain[25,26]. Recent studies have shown that LRP5 and its closely related LRP family member, LRP6, are Wnt co-receptors that are capable of interacting with several key components of the Wnt pathway, and research regarding the signaling mechanisms involved in bone regulation by LRP5 has focused on this pathway[27–29]. The LRP5 gene has 23 exons. In addition to the G171V substitution in HBM, nine disease-causing mutations in exons encoding the LRP5 extracellular domain have been identified in patients with OPPG[12,13]. We found that the Q89R and N740N polymorphisms were only associated with BMD at the femoral neck, and were not associated with BMD at the lumbar spine in this large group of postmenopausal Chinese women, which suggests that the Q89R and N740N polymorphisms can influence the attainment of peak bone mass. Although the molecular mechanisms that underlie the association of the Q89R and N740N polymorphisms of the LRP5 gene with BMD remain unclear, we consider that the Q89R and N740N or related linked polymorphisms in the region might alter LRP5 protein function and might be associated with BMD.

In conclusion, we found a significant association between the Q89R and N740N polymorphisms in the LRP5 gene and BMD at the femoral neck in postmenopausal Chinese women, but we failed to observe a significant association between the A1330V polymorphism and BMD at any site. Our findings suggest that the LRP5 gene is a candidate for the genetic determination of BMD in postmenopausal Chinese women. Further studies will be needed to determine an association between the LRP5 gene polymorphisms and the risk of osteoporosis in the general population.

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