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Association between single nucleotide polymorphisms of the osteoprotegerin gene and postmenopausal osteoporosis in Chinese women

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ABSTRACT. Osteoporosis is an important and common complex health problem, particularly in postmenopausal women. It is characterized by a reduction in bone mineral density (BMD) and a deterioration of bone microarchitecture with a consequent increase of fracture risk. The osteoprotegerin (*OPG*) gene is considered to play an important role in the pathogenesis of osteoporosis. We analyzed SNPs of the *OPG* gene and associations between these polymorphisms and BMD in 399 Chinese postmenopausal women. BMD was quantified at the lumbar spine (L_{2-4}), femoral neck, and total hip. The g.2264T>C and g.27676A>C SNPs were detected by PCR-RFLP and DNA sequencing methods. A significant association with spine BMD was found for g.27676A>C. The spine BMD value for subjects with genotype AA was significantly higher than those with genotypes GA and AA. No significant association was detected between any of the SNP marker genotypes and the other traits. We conclude that g.27676A>C in the *OPG* gene affects spine BMD and

that the C allele is associated with increased risk for osteoporosis in Chinese postmenopausal women.

Key words: Association analysis; Bone mineral density; Osteoporosis; Single nucleotide polymorphisms; Postmenopausal women; Osteoprotegerin gene

INTRODUCTION

Osteoporosis, a common complex and polygenic health problem of the postmenopausal women, is a skeletal disorder characterized by a reduction in bone mineral density (BMD) and a microarchitectural deterioration of bone tissue with a consequent increase in fracture risk (Cummings et al., 1985; Riggs and Melton, 1986, Peck, 1993; Kanis et al., 1994; Geng et al., 2007; Garcia-Unzueta et al., 2008). BMD is a complex trait that is influenced by multiple genes and environmental factors (Ohmori et al., 2002). Genetic factors play an important role in the pathogenesis of osteoporosis (Albagha and Ralston, 2006; Ferrari, 2008; Cheung et al., 2010; Hosoi, 2010; Ralston, 2010). Several genes have been found to be involved in bone mineral homeostasis, bone remodeling, and bone matrix composition, e.g., osteoprotegerin (*OPG*) (Pocock et al., 1987; Hofbauer and Schoppet, 2002; Langdahl et al., 2002; Yamada et al., 2003; Arko et al., 2002, 2005; Vidal et al., 2011), estrogen receptor (Ioannidis et al., 2002, 2004; Gennari et al., 2005), vitamin D receptor (Fang et al., 2005; Li et al., 2012), transforming growth factor b1 (Yamada, 2001), and collagen type 1a1 (Mann and Ralston, 2003). *OPG* has been considered an important candidate gene for osteoporosis, as polymorphisms in *OPG* may contribute to a genetic influence on BMD (Pocock et al., 1987; Hofbauer and Schoppet, 2002; Langdahl et al., 2002; Yamada et al., 2003; Arko et al., 2002, 2005; Vidal et al., 2011). Association analyses between *OPG* polymorphisms and BMD have been performed in osteoporosis, e.g., A163G, T245G, T950C, and G1181C (Arko et al., 2002; Langdahl et al., 2002; Ohmori et al., 2002; Jorgensen et al., 2004; Zhao et al., 2005; Kim et al., 2007; Ueland et al., 2007; Garcia-Unzueta et al., 2008; Moffett et al., 2008; Lee et al., 2010), but there have been no published results about the association between the g.2264T>C and g.27676A>C polymorphisms of the *OPG* gene and BMD in various subjects. Therefore, the objective of this study was to investigate the g.2264 T>C and g.27676A>C polymorphisms of the *OPG* gene and to evaluate their effect on BMD in Chinese women.

MATERIAL AND METHODS

Subjects

The study included 399 postmenopausal women, where 247 were primary postmenopausal osteoporosis patients (aged 49-80 years) and 152 were healthy volunteers (aged 48-81 years). Subjects suffering from diseases or taking drugs that could affect bone metabolism were excluded. All subjects were genetically unrelated ethnic Han Chinese. This study was approved by the local Ethics Committee, and informed consent was obtained from all participants.

Measurement of bone mineral density

BMD of the lumbar spine (L_{2-4}), femoral neck, and total hip was assessed using dual-energy X-ray absorptiometry (Lunar Expert 1313, Lunar Corp., USA). BMD was automatically calculated from the bone mineral content (g) and bone area (cm^2) and expressed absolutely in g/cm^2.

Genotyping test

Genomic DNA was isolated from peripheral venous blood samples according to the standard phenol/chloroform/isoamyl alcohol extraction protocol and stored at -20°C until analyzed. Based on the DNA sequences (GenBank ID: NG_012202.1) and mRNA sequences (GenBank ID: NM_002546.3) of the human *OPG* gene, 2 pairs of primers were designed using the Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA, USA) to amplify and verify candidate SNPs (Table 1). Primer sequences, annealing temperature, region, fragment sizes, and selected restriction enzymes are shown in Table 1. Polymerase chain reaction (PCR) amplifications were performed in a 20-µL reaction mixture containing 50 ng mixed DNA template, 10 pM each primer, 0.20 mM dNTPs, 2.5 mM $MgCl_2$, and 0.5 U Taq DNA polymerase (TaKaRa, Dalian, China). The PCR protocol was 95°C for 5 min followed by 32 cycles of 94°C for 30 s, annealing at the corresponding temperature (shown in Table 1) for 30 s, and 72°C for 30 s, with a final extension at 72°C for 10 min. The PCR products were separated on a 1.5% agarose gel (Promega) including 0.5 µg/mL ethidium bromide. The g.2264T>C and g.27676A>C SNPs were genotyped by the PCR-restriction fragment length polymorphism (PCR-RFLP) method. Aliquots of 5 µL PCR products were digested with 2 U restriction enzyme at 37°C for 10 h following the supplier manual. The digested products were separated by electrophoresis for 1 h at 100 V on a 2.5% agarose gel stained with ethidium bromide in 1X TAE buffer. The genotype results of allelic variation were based on the electrophoretic pattern of the restriction enzyme-treated PCR products. The PCR-amplified products of each genotype were purified using a Wizard Prep PCR purification kit and were sequenced in both directions with an ABI 3730 sequencer (Bioasia Biotechnology Co., Ltd., Shanghai, China).

Table 1. Primer pairs, PCR, and PCR-RFLP analysis used for genotyping single nucleotide polymorphisms (SNPs) in the *OPG* gene.

SNPs	Primer sequences	AT (°C)	SAF (bp)	Region	RE	RES (genotype: bp)
g.2264T>C	5'-TATCTGTGGTGCTTCTAGTTCC-3' 5'-GATGATACAGCATTATCAGAAGAT-3'	54.0	216	Intron 1	*Nla*III	TT: 216 TC: 216, 150, 66 CC: 150, 66
g.27676A>C	5'-TGTACATTGTGAAGCTGTGAAGG-3' 5'-AAGTGGGAGCAGAAGACATTG-3'	58.0	219	Exon 5	*Ava*II	AA: 219 AC: 219, 197, 22 CC: 197, 22

AT = annealing temperature; SAF = size of amplification fragment; RE = restriction enzyme; RES = size of fragments at the indicated allele after digestion of the PCR product using the respective restriction enzyme.

Statistical analysis

Allelic and genotypic frequencies in the subjects studied were calculated and the Har-

dy-Weinberg equilibrium of the mutation was determined by the chi-squared test. Quantitative data were compared by one-way ANOVA and the LSD *post hoc* test. Multiple regression and logistic regression analyses were carried out to evaluate the relationships between the variables. All data are reported as means ± SD. All statistical analyses were performed with the Statistical Package for Social Sciences software (SPSS, Windows version release 15.0; SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered to be statistically significant.

RESULTS

SNP identification and genotyping

In this study, the g.2264T>C and g.27676A>C SNPs were identified by PCR-RFLP and DNA sequencing methods, including T→C mutation at position 2264 and A→C mutation [resulting in phenylalanine (Phe) to valine (Val) amino acid replacement, p.Phe371Val] at position 27676 of the human *OPG* gene, respectively, in intron 1 and exon 5 (reference sequences NG_012202.1, NM_002546.3, and NP_002537.3). The PCR product of g.2264T>C was digested with *Nla*III enzyme and divided into 3 genotypes, TT (216 bp), TC (216, 150, and 66 bp), and CC (150 and 66 bp) (Table 1). The PCR product of g.27676 A>C was digested with the *Ava*II enzyme, and the 3 possible genotypes were defined by 3 distinct banding patterns: AA (219 bp), AB (219, 197, and 22 bp), and BB (197 and 22 bp) (Table 1).

Frequencies of allele and genotype

The allelic and genotypic frequencies of the g.2264T>C and g.27676A>C polymorphisms are shown in Table 2. Allele T and allele A were predominant alleles, and the genotype TT and AA frequencies were the highest in the subjects studied, respectively, in g.2264T>C (Table 2) and g.27676A>C (Table 3). The result of the chi-squared test for g.2264T>C and g.27676A>C in the populations studied indicated that the polymorphism sites were in Hardy-Weinberg equilibrium ($P > 0.05$).

Table 2. Genotypic and allelic frequencies of the *OPG* gene g.2264T>C polymorphism in the groups studied.

Groups	Genotypic frequencies (N)			Allelic frequencies (N)	
	TT	TC	CC	T	C
Case group (N = 247)	0.5223 (129)	0.3846 (95)	0.0931 (23)	0.7146 (353)	0.2854 (141)
Control group (N = 152)	0.4868 (74)	0.3947 (60)	0.1184 (18)	0.6842 (208)	0.3158 (96)
		$\chi^2 = 0.8432$, P = 0.6560		$\chi^2 = 0.831$, P = 0.362	

Table 3. Genotypic and allelic frequencies of the *OPG* gene g.27676A>C polymorphism in the groups studied.

Groups	Genotypic frequencies (N)			Allelic frequencies (N)	
	AA	AC	CC	A	C
Case group (N = 247)	0.5628 (139)	0.3603 (89)	0.0769 (19)	0.7429 (367)	0.2571 (127)
Control group (N = 152)	0.5461 (83)	0.3487 (53)	0.1053 (16)	0.7204 (219)	0.2796 (85)
		$\chi^2 = 0.9445$, P = 0.6236		$\chi^2 = 0.4892$, P = 0.4843	

Association of *OPG* gene polymorphisms with BMD

Age, weight, height, body mass index, spine BMD, femoral neck hip BMD, and total hip BMD in each genotype group were given as means ± SD (Tables 4 and 5). The gene-specific SNP marker association analysis indicated a significant association of g.27676A>C with spine BMD. The spine BMD of subjects with genotype AA was significantly higher than that of genotypes GA and AA (Table 5). However, no significant association was detected between any of the SNP marker genotypes and other traits measured in this study (Tables 4 and 5).

Table 4. Characteristics of the total group of subjects according to the g.2264T>C genotype.

Genotype	TT	TC	CC	P
Number (%)	203 (0.5088)	155 (0.3885)	41 (0.1028)	-
Age (years)	61.8 ± 7.5	62.1 ± 7.9	62.4 ± 6.5	0.341
Weight (kg)	60.7 ± 7.1	61.5 ± 6.5	62.5 ± 5.5	0.224
Height (cm)	159 ± 7.7	160 ± 6.6	161 ± 5.8	0.453
BMI	23.4 ± 3.51	23.5 ± 3.39	23.7 ± 3.43	0.469
Spine BMD (g/cm^2)	0.898 ± 0.124	0.848 ± 0.129	0.833 ± 0.125	0.053
Femoral neck hip BMD (g/cm^2)	0.754 ± 0.105	0.696 ± 0.109	0.694 ± 0.110	0.061
Total hip BMD (g/cm^2)	0.840 ± 0.109	0.826 ± 0.111	0.823 ± 0.114	0.072

BMI = body mass index; BMD = bone mineral density. Data are reported as means ± SD (BMD values adjusted by age and weight).

Table 5. Characteristics of the total group of subjects according to the g.27676A>C genotype.

Genotype	AA	AC	CC	P
Number (%)	222 (0.5564)	142 (0.3559)	35 (0.0877)	-
Age (years)	61.8 ± 7.5	62.6 ± 8.1	62.3 ± 6.1	0.343
Weight (kg)	61.7 ± 7.7	62.2 ± 6.1	62.3 ± 5.5	0.556
Height (cm)	159 ± 7.4	160 ± 6.7	161 ± 6.1	0.448
BMI	23.5 ± 3.48	23.7 ± 3.33	23.9 ± 3.21	0.556
Spine BMD (g/cm^2)	0.918 ± 0.132	0.849 ± 0.129	0.831 ± 0.121	0.041
Femoral neck hip BMD (g/cm^2)	0.743 ± 0.104	0.697 ± 0.107	0.689 ± 0.108	0.065
Total hip BMD (g/cm^2)	0.844 ± 0.111	0.827 ± 0.176	0.821 ± 0.109	0.069

Data are reported as means ± SD (BMD values adjusted by age and weight). For abbreviations, see legend to Table 4.

DISCUSSION

In the present study, we investigated the role of *OPG* in the pathogenesis of osteoporosis by association analysis. The results showed that the g.27676A>C SNP polymorphism in exon 5 was associated with spine BMD in women. Subjects with genotype AA had significantly higher BMD compared to genotypes AC and CC (Table 5, P = 0.041), and the AA genotype had a higher femoral neck hip BMD and total hip BMD than genotypes AC and CC, almost reaching a significant level (Table 4, P = 0.065 and P = 0.069). The C allele could be a risk factor for osteoporosis in Chinese postmenopausal women. As for g.2264T>C SNP in intron 2, the TT genotype had a higher spine BMD, femoral neck hip BMD, and total hip BMD than genotypes TC and CC, almost reaching a significant level (Table 4, P = 0.053, P = 0.061, and P = 0.072, respectively). Although introns are not coding sequences, there is constant evidence to prove that introns play an important role in regulating mRNA splicing, transcription, and gene expression and regulation (Nott et al., 2003; Zheng et al., 2011; Yuan et al., 2012).

Whether the detected g.2264T>C locus in the *OPG* gene intron 6 affects gene expression and regulation needs to be determined. Previous studies have found an association between several SNPs and BMD, e.g., A163G, T245G, T950C, and G1181C, which is consistent with our findings that polymorphisms in the *OPG* gene may contribute to a genetic influence on BMD in osteoporosis (Arko et al., 2002; Langdahl et al., 2002; Ohmori et al., 2002; Jorgensen et al., 2004; Zhao et al., 2005; Kim et al., 2007; Ueland et al., 2007; Garcia-Unzueta et al., 2008; Moffett et al., 2008; Lee et al., 2010). How these polymorphisms affect BMD still remains poorly understood. Further studies are necessary to elucidate the underlying molecular mechanism and pathophysiology.

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