



Association of the g.27563G>A osteoprotegerin genetic polymorphism with bone mineral density in Chinese women

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ABSTRACT. Osteoporosis is a common multifactorial disease in postmenopausal women. This study aimed to investigate the association of the g.27563G>A osteoprotegerin (*OPG*) genetic polymorphism with bone mineral density (BMD) and osteoporosis. A case-control study was carried out with 435 osteoporosis postmenopausal women cases and 442 age-matched healthy controls. The BMD at the femoral neck hip, lumbar spine (L₂₋₄), and total hip were assessed by Norland XR-46 dual-energy X-ray absorptiometry. The genotypes of the g.27563G>A genetic polymorphism were detected by created restriction site-polymerase chain reaction and verified by DNA sequencing methods. We detected that the g.27563G>A genetic polymorphism was a non-synonymous mutation that resulted in an arginine (Arg) to glutamine (Gln) amino acid replacement

(p.Arg333Gln). Significant differences were found in the BMD of the femoral neck hip, lumbar spine (L₂₋₄), and total hip among different genotypes of the g.27563G>A genetic polymorphism. Subjects with the genotype GG had significantly higher BMD values than those with genotypes GA and AA ($P < 0.05$). Our data indicated that the A allele of the g.27563G>A genetic polymorphism in *OPG* could be associated with lower BMD values in the Chinese postmenopausal women evaluated, and that it might be an increased risk factor for osteoporosis.

Key words: Bone mineral density; Osteoporosis; Osteoprotegerin gene; Genetic polymorphism; Risk factor; Chinese women

INTRODUCTION

Osteoporosis is a common and complex disease that is characterized by a reduction in bone mineral density (BMD) and a microarchitecture deterioration of bone tissue with a consequent increase of fracture risk (Cummings et al., 1985; Riggs and Melton III, 1986; Kanis et al., 1994; Geng et al., 2007; Garcia-Unzueta et al., 2008; Li et al., 2012; Woo et al., 2012). Osteoporosis is a major health problem in postmenopausal women. Recent large epidemiological studies have reported that low BMD is a major risk factor for osteoporosis (Nguyen et al., 2000; Lee et al., 2010; Ozbas et al., 2012) and that BMD is a complex trait that is determined by metabolic, genetic, and environmental factors. Several studies have shown that genetic factors play key roles in the development of osteoporosis (Nguyen et al., 2000; Ohmori et al., 2002; Albagha and Ralston, 2006; Ferrari, 2008; Cheung et al., 2010; Hosoi, 2010; Lee et al., 2010; Ralston, 2010; Feng et al., 2012; Ozbas et al., 2012; Woo et al., 2012; Zhang et al., 2013). Various candidate genes have been reported to influence BMD and osteoporosis, such as the osteoprotegerin (*OPG*) gene (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; Feng et al., 2012; Hussien et al., 2013; Zhang et al., 2013), the transforming growth factor β 1 (TGF β 1) gene (Yamada, 2001), the collagen type 1a1 (COL1A1) gene (Mann and Ralston, 2003; Falcon-Ramirez et al., 2011), the estrogen receptor alpha (ER- α) gene (Kurt et al., 2012), and the vitamin D receptor (VDR) gene (Fang et al., 2005; Jakubowska-Pietkiewicz et al., 2012; Kurt et al., 2012; Li et al., 2012; Hussien et al., 2013; Horst-Sikorska et al., 2013). The *OPG* gene was first cloned and characterized by Morinaga et al. (1998), and has since been considered an important candidate gene for mediating the genetic influence on osteoporosis. In humans, some genetic polymorphisms of *OPG*, for example, A163G, T950C, T245G, G1181C, C21775T, G23276A, and T23367C, have been reported to influence BMD and osteoporotic fractures (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; Feng et al., 2012; Zhang et al., 2013). However, the potential association of the g.27563G>A genetic polymorphism of *OPG* with BMD and osteoporosis has not yet been investigated. In the present study, we aimed to examine the g.27563G>A genetic polymorphism and investigated its effect on BMD and osteoporosis based on a case-control study.

MATERIAL AND METHODS

Subjects

A case-control study was carried out with 435 osteoporosis postmenopausal women cases and 442 age-matched healthy controls. All subjects were of Chinese Han nationality and lived in Dalian city. Those suffering diseases or taking drugs that are known to affect skeletal homeostasis or interfere with bone metabolism were excluded. This study was approved by the local Ethics Committee. Informed consent forms were obtained from all subjects included in the study.

Measurement of BMD

BMD was quantified using Norland XR-46 dual-energy X-ray absorptiometry (Norland Coopersurgical Corp., USA) (Tothill et al., 1999) at lumbar spine (L_{2-4}), femoral neck hip, and total hip. The BMD value was calculated from bone mineral content (g) and bone area (cm^2), and then expressed as g/cm^2 . All BMD values were adjusted by age, weight, and height.

Polymerase chain reaction (PCR) and genotyping

Genomic DNA was isolated from peripheral venous blood using the Qiagen method (Hilden, Germany), and stored at -80°C until analyzed. The PCR primers were constructed using the Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA, USA). The PCR primers sequences, amplified fragment region, annealing temperature, and genotype size are shown in Table 1. The PCRs were performed in a total volume of 20 μL containing 50 ng mixed DNA template, 10 pM-each primer, 0.20 mM dNTP, 2.5 mM MgCl_2 , and 0.5 U Taq DNA polymerase (TianGen Biotech Inc., China). The PCR cycling conditions consisted of an initial denaturation step for 5 min at 94°C , followed by 32 cycles for 30 s at 94°C , 30 s at 58.5°C , and 30 s at 72°C , and a final extension for 5 min at 72°C . We detected the g.27563G>A genetic polymorphism through the created restriction site (CRS)-PCR method. Following the supplier manual, 5- μL aliquot PCR-amplified products were digested with 2 U *TaqI* restriction enzymes (MBI Fermentas, St. Leon-Rot, Germany) at 37°C for 10 h, which were separated by electrophoresis, and observed under UV light. DNA sequencing (ABI3730xl DNA Analyzer, Applied Biosystems, Foster City, CA, USA) was utilized to verify the accuracy of the genotype results obtained from the CRS-PCR method.

Statistical analyses

The chi-squared (χ^2) test was utilized to identify significant departures from Hardy-Weinberg equilibrium (HWE) for allele and genotype distributions. All data are reported as means \pm standard deviation (SD). The quantitative data were analyzed by one-way analysis of variance (ANOVA) and unpaired Student *t*-tests. The association analyses between different variables were evaluated by multiple regression analyses. A P value less than 0.05 was considered to be statistically significant. All statistical analyses were performed with the Statistical Package for Social Sciences software (SPSS 15.0; SPSS Inc., Chicago, IL, USA).

RESULTS

Genotyping of the *OPG* genetic polymorphism

A novel genetic polymorphism (g.27563G>A) in *OPG* was detected by the CRS-PCR method in the present study. The amplified PCR products were digested with *TaqI* restriction enzyme and divided into three genotypes: GG (190 and 21 bp), GA (211, 190, and 21 bp), and AA (211 bp) (Table 1). Results of sequence analyses suggested that this genetic polymorphism was a non-synonymous mutation in exon 5 at position 27,563 of the *OPG* gene that was caused by a G→A mutation, and resulted in an arginine (Arg) to glutamine (Gln) amino acid replacement (p.Arg333Gln, reference sequences GenBank IDs: NG_012202.1, NM_002546.3, NP_002537.3).

Table 1. PCR and CRS-PCR analysis used for g.27563G>A genetic polymorphism in the *OPG* gene.

Primer sequences	Annealing temperature (°C)	PCR amplification fragment (bp)	Region	Restriction enzyme	Genotype (bp)
5'-CTGAAGCTGCTCAGTTTGTGTC-3' 5'-CTGATTGGACCTGGTTACCTATC-3'	57.7	211	Exon 5	<i>TaqI</i>	GG: 190, 21 GA: 211, 190, 21 AA: 211

PCR = polymerase chain reaction; CRS-PCR = created restriction site-PCR. Underlined nucleotide marks nucleotide mismatches enabling the use of the selected restriction enzymes for discriminating sequence variations.

Allele and genotype frequencies

The allele and genotype frequencies for the g.27563G>A genetic polymorphism in osteoporosis cases and healthy controls are shown in Table 2. The allele frequencies in osteoporosis cases (G, 64.60%; A, 35.40%) were significantly different ($\chi^2 = 4.4666$, $P = 0.0346$) from those of healthy controls (G, 69.34%; A, 30.66%). The genotype frequencies in osteoporosis cases (GG, 43.68%; GA, 41.84%; AA, 14.48%) were significantly different ($\chi^2 = 6.3974$, $P = 0.0408$) from those in healthy controls (GG, 47.74%; GA, 43.21%; AA, 9.05%). Results from the χ^2 test indicated that the genotype distributions were in line with HWE for the studied subjects ($P > 0.05$).

Table 2. Allelic and genotypic frequencies of g.27563G>A genetic polymorphism in the populations studied.

Groups	Genotypic frequencies (%)			Allelic frequencies (%)		P	χ^2
	GG	GA	AA	G	A		
Case group (N = 435)	190 (43.68)	182 (41.84)	63 (14.48)	562 (64.60)	308 (35.40)	0.2059	3.1612
Control group (N = 442)	211 (47.74)	191 (43.21)	40 (9.05)	613 (69.34)	271 (30.66)	0.9424	0.1186
Total (N = 877)	401 (45.73)	373 (42.53)	103 (11.74)	1175 (66.99)	579 (33.01)	0.5249	1.2891
	$\chi^2 = 6.3974$, $P = 0.0408$			$\chi^2 = 4.4666$, $P = 0.0346$			

Association analysis between the *OPG* genetic polymorphism and BMD

The characteristics of cases and controls are shown in Table 3, including age, weight, height, body mass index, femoral neck hip BMD, spine BMD, and total hip BMD. Our data indicated that there were significant differences in femoral neck hip BMD, spine BMD, and

total hip BMD among different genotypes in the studied subjects; subjects with the GG genotype showed higher BMD values compared to those with the GA and AA genotypes ($P < 0.05$; Table 3).

Table 3. Characteristics of g.27563G>A genetic polymorphism in the total population studied.

Genotype	GG	GA	AA	P
Number (%)	401 (45.73)	373 (42.53)	103 (11.74)	-
Age (years)	62.7 ± 7.9	63.3 ± 7.1	63.8 ± 8.1	0.445
Height (cm)	161 ± 8.2	162 ± 8.8	165 ± 6.9	0.336
Weight (kg)	61.1 ± 6.6	61.9 ± 7.1	62.6 ± 7.2	0.189
BMI	23.3 ± 3.19	23.6 ± 3.18	23.9 ± 3.45	0.227
Femoral neck hip BMD (g/cm ²)	0.751 ± 0.028	0.687 ± 0.112	0.657 ± 0.129	0.038
Spine BMD (g/cm ²)	0.951 ± 0.106	0.865 ± 0.111	0.841 ± 0.185	0.029
Total hip BMD (g/cm ²)	0.892 ± 0.116	0.837 ± 0.135	0.812 ± 0.284	0.037

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

DISCUSSION

Osteoporosis, a major health problem in postmenopausal women, is a polygenic and multifactorial disease caused by the combined effects of genetic and environmental factors (Ohmori et al., 2002; Zhao et al., 2005). It is generally accepted that genetic factors play important roles in the pathogenesis of osteoporosis (Nguyen et al., 2000; Ohmori et al., 2002; Albagha and Ralston, 2006; Ferrari, 2008; Cheung et al., 2010; Hosoi, 2010; Lee et al., 2010; Ralston, 2010; Feng et al., 2012; Ozbas et al., 2012; Woo et al., 2012; Zhang et al., 2013). Because of the potentially important role of *OPG* in controlling bone resorption, the *OPG* gene is a candidate for mediating the genetic influence on BMD and osteoporosis (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; Feng et al., 2012; Hussien et al., 2013; Zhang et al., 2013). In this study, we assessed the relevance of genetic polymorphisms of *OPG* in relation to BMD and osteoporosis through an association analysis. We firstly investigated the g.27563G>A genetic polymorphism in exon 5 of *OPG* using the CRS-PCR method. We then evaluated the potential association of this genetic polymorphism with BMD and osteoporosis. Results from this study demonstrated a statistically significant association between this genetic polymorphism and BMD and osteoporosis in Chinese postmenopausal women; subjects with the GG genotype had significantly higher BMD values compared to those with genotypes GA and AA ($P < 0.05$; Table 3). Therefore, the A allele could be an increased risk factor for BMD and osteoporosis. Several similar studies have reported that genetic polymorphisms of *OPG*, such as A163G, T245G, T950C, G1181C, C21775T, G23276A, and T23367C, have potential associations with BMD and 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; Feng et al., 2012; Zhang et al., 2013). Results from these observations are consistent with our findings that the genetic polymorphisms of *OPG* may play key roles in contributing to the genetic influence on BMD and osteoporosis (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; Feng et al., 2012; Hussien et al., 2013; Zhang et al., 2013). According to the results of the sequence analyses, we found that the g.27563G>A

genetic polymorphism is a non-synonymous mutation that resulted in an Arg to Gln amino acid replacement. This substitution might alter the function of the *OPG* protein. In addition, this genetic polymorphism may be linked to other non-synonymous genetic polymorphisms, for example, Lysine (Lys) 3 Asparagine (Asn), Isoleucine (Ile) 184 Methionine (Met), and Threonine (Thr) 154Met, which have all been shown to be significantly associated with the risk of BMD and osteoporosis (Zhao et al., 2005; Feng et al., 2012; Zhang et al., 2013). Our study demonstrated that, for the g.27563G>A genetic polymorphism, people carrying the GG genotype may have a lower risk of developing osteoporosis compared to those with the GA and AA genotypes (Table 3).

In conclusion, the present study, for the first time to the best of our knowledge, identified that the *OPG* g.27563G>A genetic polymorphism could influence BMD and osteoporosis in Chinese postmenopausal women. Epidemiological investigations in other ethnicities or with a larger number of subjects are necessary to confirm our findings and to clarify the underlying molecular mechanism.

Conflicts of interest

The authors declare no conflict of interest.

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