Association of the g.19074G>A genetic variant in the osteoprotegerin gene with bone mineral density in Chinese postmenopausal women

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ABSTRACT. Primary osteoporosis is a common health problem in postmenopausal women. This study aimed to detect the association of the g.19074G>A genetic variant in the osteoprotegerin gene (*OPG*) with bone mineral density (BMD) and primary osteoporosis. The created restriction site-polymerase chain reaction method was used to investigate the g.19074G>A genetic variant. The BMD of the femoral neck hip, lumbar spine (L_{2-4}), and total hip were assessed by dual-energy X-ray absorptiometry (DEXA) in 856 unrelated Chinese postmenopausal women. We found significant differences in the BMDs of the femoral neck hip, lumbar spine (L_{2-4}), and total hip among different genotypes; individuals with the GG genotype had significantly

higher BMDs than those with the GA and AA genotypes (P < 0.05). Our results indicated that the A allele was an increased risk factor for primary osteoporosis and the g.19074G>A genetic variant of the *OPG* gene was associated with BMD and primary osteoporosis in Chinese postmenopausal women.

Key words: Bone mineral density; Genetic variant; Osteoprotegerin gene; Primary osteoporosis; Risk factor

INTRODUCTION

Primary osteoporosis is a common polygenic health problem, particularly in post-menopausal women. It is a multifactorial disorder characterized by a reduction in bone mineral density (BMD) and a deterioration of bone microarchitecture with a consequent increase of fracture risk (Cummings et al., 1985; Riggs and Melton, 1986; Peck et al., 1993; Kanis et al., 1994; Geng et al., 2007; Garcia-Unzueta et al., 2008; Li et al., 2012; Woo et al., 2012). Genetic factors play important roles in the pathogenesis of primary osteoporosis (Albagha and Ralston, 2006; Ferrari, 2008; Cheung et al., 2010; Hosoi, 2010; Ralston, 2010; Feng et al., 2012; Woo et al., 2012; Zhang et al., 2013). Evidence suggests that low BMD has high heritability. Low BMD is a major risk factor for primary osteoporosis (Nguyen et al., 2000; Lee et al., 2010; Ozbas et al., 2012). To date, several studies have reported that some functional genes, for example, 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 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), and the estrogen receptor alpha (ERalpha) gene (Kurt et al., 2012), affected BMD and primary osteoporosis. Among these candidate genes, *OPG* is one of the most important. The potential association of the A163G, T245G, T950C, G1181C, C21775T, G23276A, and T23367C genetic variants of the *OPG* gene with BMD and primary osteoporosis have been analyzed (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), and the results indicated that these genetic variants contributed to BMD and primary osteoporosis. However, the potential association between the g.19074G>A genetic variant of *OPG* and BMD and primary osteoporosis have not been reported. Therefore, the objective of this study was to detect this genetic variant and to assess its influence on BMD and primary osteoporosis.

MATERIAL AND METHODS

Subjects

In the present study, 425 Chinese postmenopausal osteoporosis women and 431 age-matched healthy postmenopausal women were enrolled from the Affiliated East Hospital of Tongji University (Shanghai, China). All subjects were of Chinese Han nationality and lived

in Shanghai City. Individuals with present or past history of diseases that might affect bone metabolism were excluded. All participants completed informed consent forms. This study was approved by the Ethics Committee of the Affiliated East Hospital of Tongji University (Shanghai, China).

BMD measurement

BMDs of the femoral neck hip, lumbar spine (L_{2-4}), and total hip were measured by dual-energy X-ray absorptiometry (DEXA) (Lunar Expert 1313; Lunar Corp.; USA). The BMD value was automatically calculated from bone mineral content (g) and bone area (cm^2), and expressed as g/cm^2.

Genotyping

Peripheral venous blood was collected from each subject. Genomic DNA was isolated from blood using a DNA isolation kit (Invitrogen; Carlsbad, CA, USA). The primers for polymerase chain reaction (PCR) were designed through the Primer Premier 5.0 software (Premier Biosoft International; Palo Alto, CA, USA). Table 1 shows the PCR primers sequences, annealing temperature, PCR fragment size and region, and genotype size. The PCR was performed in 20 µL mixtures, including 50 ng mixed DNA template, 10 pM of each primer, 0.20 mM dNTP, 2.5 mM $MgCl_2$, and 0.5 U Taq DNA polymerase (TaKaRa; Dalian, China). The protocol of PCR amplification was carried out at 95°C for 5 min followed by 32 cycles of 94°C for 30 s, 59.5°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 5 min. The created restriction site-PCR (CRS-PCR) method was used to investigate the g.19074G>A genetic variant. With one of the primers containing a nucleotide mismatch, the CRS-PCR method enabled the use of restriction enzymes for discriminating sequence variants (Yuan et al., 2012; 2013a, b). Five microliters of PCR amplified products were digested with 2 units selected *Mst*I restriction enzymes (MBI Fermentas; St. Leon-Rot, Germany) at 37°C for 10 h and separated by electrophoresis. In order to confirm the genotype accuracy of CRS-PCR method, about 10% random samples were investigated by DNA sequencing (ABI3730xl DNA Analyzer; Applied Biosystems, Foster City, CA, USA).

Table 1. PCR and CRS-PCR analysis for g.19074G>A genetic variant of the *OPG* gene.

Primer sequences	Annealing temperature (°C)	PCR Amplification fragment (bp)	PCR Amplification region	Restriction enzyme	Genotype (bp)
5'-TCTATACTGCAGCCCCGTG<u>C</u>-3' 5'-CAACTATCTGACTTTGCATGATCC-3'	61.0	215	Exon2	*Mst*I	GG: 195, 20 GA: 215

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.

Statistical analyses

The chi-squared (χ^2) test was utilized to evaluate the Hardy-Weinberg equilibrium (HWE) for genotypic distributions. One-way analysis of variance (ANOVA) and the unpaired

Student *t*-tests were used to evaluate the quantitative data. All data are reported as means ± standard deviation (SD). The associations of variables were detected by multiple regression analyses. $P < 0.05$ was considered to be statistically significant level. All statistical analyses were analyzed with the Statistical Package for Social Sciences software (SPSS 14.0; SPSS Inc.; Chicago, IL, USA).

RESULTS

OPG genetic variant identification

A total of 856 subjects were recruited for this study. We detected a novel genetic variant (g.19074G>A) in the *OPG* gene for the subjects studied using the CRS-PCR method. The sequence analyses suggested that this genetic variant is a non-synonymous mutation in exon2 at position 19074 of the *OPG* gene that is caused by a G→A mutation, and resulted in a cysteine (Cys) to tyrosine (Tyr) amino acid replacement (p.Cys87Tyr; reference sequence GenBank IDs: NG_012202.1, NM_002546.3, NP_002537.3). The PCR amplified products were digested with the *Mst*I selected restriction enzyme and divided into three genotypes: GG (195 bp and 20 bp), GA (215 bp, 195 bp, and 20 bp), and AA (215 bp) (Table 1).

Allelic and genotypic distributions

All three possible genotypes of the g.19074G>A genetic variant for the populations studied were found. The allelic and genotypic distributions of this genetic variant in primary osteoporosis cases and healthy controls are shown in Table 2. The G allele and the GG genotype were predominant in the subjects studied. The results of the χ^2 test suggested that the distributions of genotype conformed to HWE ($P > 0.05$).

Table 2. Genotypic and allelic frequencies of g.19074G>A genetic variant in the subjects studied.

Groups	Genotypic frequencies (%)			Allelic frequencies (%)		χ^2	P
	GG	GA	AA	G	A		
Case group (N = 425)	183 (43.06)	174 (40.94)	68 (16.00)	540 (63.53)	310 (36.47)	5.7670	0.0559
Control group (N = 431)	206 (47.80)	180 (41.76)	45 (10.44)	592 (68.68)	270 (31.32)	0.3695	0.8313
Total (N = 856)	389 (45.44)	354 (41.36)	113 (13.20)	1132 (66.12)	580 (33.88)	5.0666	0.0794
	$\chi^2 = 6.1013$, P = 0.0473			$\chi^2 = 5.0634$, P = 0.0244			

Association analyses

The age, height, weight, body mass index (BMI), femoral neck hip BMD, spine BMD, and total hip BMD in each genotype group are shown in Table 3. The BMD values were adjusted by age, height, and weight. We found significant differences in the BMDs of the femoral neck hip, lumbar spine (L_{2-4}), and total hip among different genotypes in the studied subjects; individuals with the GG genotype had significantly higher BMDs than those with the GA and AA genotypes ($P < 0.05$, Table 3).

Table 3. Characteristics of g.19074G>A genetic variant in the total subjects.

Genotype	GG	GA	AA	P
Number (%)	389 (45.44)	354 (41.36)	113 (13.20)	-
Age (years)	61.8.3 ± 7.6	62.9 ± 8.1	63.2 ± 7.8	0.411
Height (cm)	161 ± 7.2	162 ± 7.8	164 ± 6.8	0.256
Weight (kg)	61.2 ± 6.6	61.6 ± 6.9	62.2 ± 7.7	0.321
BMI	23.1 ± 3.11	23.2 ± 3.22	23.5 ± 3.53	0.318
Femoral neck hip BMD (g/cm²)	0.752 ± 0.183	0.689 ± 0.148	0.667 ± 0.213	0.035
Spine BMD (g/cm²)	0.956 ± 0.137	0.876 ± 0.111	0.842 ± 0.219	0.046
Total hip BMD (g/cm²)	0.895 ± 0.117	0.831 ± 0.142	0.820 ± 0.289	0.035

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

DISCUSSION

Primary osteoporosis is a common health disease in postmenopausal women and is caused by the effects combined of genetic and environmental factors. Genetic factors play key roles in the development of primary osteoporosis. Recent studies indicated that the *OPG* gene is an important candidate gene influencing BMD and primary 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). Several genetic variants of the *OPG* gene have been analyzed and the results suggested that these genetic variants could mediate the effects of BMD and primary osteoporosis (Pocock et al., 1987; Hofbauer and Schopppet, 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 the present study, one novel genetic variant (g.19074G>A) in the *OPG* gene was detected using CRS-PCR and verified with DNA sequencing methods. Our data indicated a significant association between this genetic variant and BMD and primary osteoporosis in Chinese post-menopausal women; individuals with the GG genotype had significantly higher BMD values than those with GA and AA genotypes (P < 0.05, Table 3). Results from this study suggested that the A allele might confer an increased risk for BMD and primary osteoporosis in Chinese postmenopausal women. To date, several similar studies have reported potential associations of many genetic variants of the *OPG* gene (for example, A163G, T245G, T950C, G1181C, C21775T, G23276A, and T23367C) with BMD and primary osteoporosis. Results from these observations are consistent with our conclusion that the genetic variants of the *OPG* gene may contribute to BMD and primary 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). Our results could provide more evidence to explain the role of the *OPG* gene in the development of primary osteoporosis. Several other non-synonymous genetic variants, such as Lysine (Lys)3 Asparagine (Asn), Isoleucine (Ile)184 Methionine (Met) and Threonine (Thr)154Met, have been significantly associated with the risk of BMD and primary osteoporosis and were shown to influence the function of the OPG protein (Zhao et al., 2005; Feng et al., 2012; Zhang et al., 2013). The g.19074G>A genetic variant of the *OPG* gene might be linked to these non-synonymous genetic variants and have similar functions in the development of primary osteoporosis. Functional studies on larger, different populations with respect to the g.19074G>A variant and other genetic variants spanning the whole *OPG* gene region

are necessary to clarify the underlying molecular mechanisms and pathophysiology of this association.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

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