Research paper

Association between g.19163A>G and g.23298T>C genetic variants of the osteoprotegerin gene and bone mineral density in Chinese women

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ABSTRACT

OBJECTIVE: Osteoporosis is a serious and common health issue of considerable complexity among postmenopausal women. The osteoprotegerin gene (*OPG*) is considered to play an important role in the pathogenesis of osteoporosis. The objective of this study was to detect single nucleotide polymorphisms (SNPs) in the *OPG* gene and assess the association between bone mineral density (BMD) and SNPs in postmenopausal women. **METHODS:** BMD was measured at the lumbar spine (L_{2-4}), neck, and total hip by dual energy X-ray absorptiometry (DEXA). The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), created restriction site-PCR (CRS-PCR), and DNA sequencing methods were used to identify the g.19163A>G and g.23298T>C polymorphisms and genotypes in 739 Chinese postmenopausal women. **RESULTS:** Our data suggest that g.19163A>G was significantly associated with adjusted spine BMD, neck hip BMD, and total hip BMD. Subjects with genotype AA had significantly higher BMD value than those of genotypes AG and GG (P <0.05). We failed to detect any statistically significant association between g.23298T>C and adjusted spine BMD and neck hip BMD, while it almost reached a significant association with the adjusted total hip BMD (P = 0.058). **DISCUSSION:** These findings indicate that the *OPG* gene is related to BMD and osteoporosis in Chinese postmenopausal women.

Key words: Bone mineral density, Osteoporosis, Osteoprotegerin gene, Postmenopausal, Single nucleotide polymorphisms

INTRODUCTION

Osteoporosis is a common polygenic health prob-

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lem of considerable complexity and importance, particularly in postmenopausal women. It is characterized by a reduction in bone mineral density (BMD) and a microarchitectural deterioration of bone tissue with a consequent increase of fracture risk.[1-6] Evidence from previous studies suggested that BMD is a complex trait that is caused by multiple genes and environmental factors.[7] A large number of studies have demonstrated that genetic factors may play a significant

role in the pathogenesis of osteoporosis.[8-13] To date, several genes have been verified as being involved in bone remodeling, bone mineral homeostasis, and bone matrix composition, including osteoprotegerin (*OPG*),[14-23] vitamin D receptor (*VDR*),[21,24-28] estrogen receptor (*ESR*),[29-31] collagen type 1a1 (*COL1A1*),[32] and transforming growth factor b1 (*TGFB1*).[33] Previous studies indicated that the *OPG* gene is considered to be one of the most important candidate genes in the pathogenesis of osteoporosis.[14-23] The single nucleotide polymorphisms (SNPs) in the *OPG* gene may contribute to genetic effects on osteoporosis and BMD.[14-20,22,23] The correlation analyses between the *OPG* genetic variants and BMD have been analyzed in osteoporosis, such as A163G, T245G, T950C and G1181C.[6,7,15,19,22,23,34-39] However, the association of g.19163A>G and g.23298T>C SNPs of the *OPG* gene with osteoporosis and BMD have not previously been published. Therefore, the aim of this study was to evaluate the association between these two SNPs and osteoporosis and BMD in postmenopausal women.

MATERIALS AND METHODS

Subjects

A total of 739 postmenopausal women were enrolled in this study, including 367 primary postmenopausal osteoporosis patients (aged 48-79 years) and 372 healthy controls (aged 47-79 years). All subjects were genetically unrelated and of the Chinese Han population. Subjects were excluded when suffering from diseases or taking drugs that could affect bone metabolism. The present study was approved by the local ethics committee and all participants gave their informed consent.

Bone mineral density measurement

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

Genotyping of genetic variants

Genomic DNA was isolated from peripheral venous blood samples using the QIAamp DNA Blood Mini Kit (QIAGEN GmbH, Germany), in accordance with the supplier manual, and then stored at -20°C until analyzed. Polymerase chain reaction (PCR) primers were designed using Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA) based on the DNA sequences (GenBank ID: NG_012202.1) and mRNA sequences (GenBank ID: NM_002546.3) of the human *OPG* gene. Primer sequences, annealing temperature, region, and fragment sizes are presented in Table 1. PCR amplifications were carried out 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 protocol of PCR was 95°C for 5 minutes followed by 32 cycles of 94°C for 30 seconds, annealing at the corresponding

Table 1. PCR, PCR-RFLP and CRS-PCR analysis used for genotyping SNPs in *OPG* gene

SNPs	Primer sequences	Annealing temperature (°C)	PCR amplification fragment (bp)	Region	Restriction enzyme	Genotype (bp)
g.19163A>G	5′-GCTGCACATTGACACGTACCAGC-3′	66.5	256	Exon2	*Sac*I	AA:256
	5′-CAGCAAAGTGGAAGACCGTGTGC-3′					AG:256,183,73
						GG:183,73
g.23298T>C	5′-CTGGAGGCCTTGTGTTCAACTC-3'	62.3	210	Exon3	*Taq*I	TT: 190,20
	5′-CGTCATCTAAAGCACCCTGT<u>C</u>G-3'					TC:210,190,20
						CC:210

Note: PCR: polymerase chain reaction, PCR-RFLP: PCR-restriction fragment length polymorphism, CRS-PCR: created restriction site-PCR, SNPs: single nucleotide polymorphisms.

Underlined nucleotides mark nucleotide mismatches enabling the use of the selected restriction enzymes for discriminating sequence variations.

temperature (shown in Table 1) for 30 seconds, and 72˚C for 30 seconds, with a final extension at 72˚C for 10 minutes. The PCR amplified products were separated on a 2.5% agarose gel including ethidium bromide. The g.19163A>G variant was genotyped by the PCR-restriction fragment length polymorphism (PCR-RFLP) method. The g.23298T>C variant was detected by the created restriction site-PCR (CRS-PCR) method with one of the primers containing a nucleotide mismatch, which enables the use of restriction enzymes for discriminating sequence variations.[40-44] Aliquots of 5 μL PCR amplified products were digested with 2 U selected restriction enzymes (MBI Fermentas, St. Leon-Rot, Germany) at 37˚C for 10 h following the manufacturer's instructions (Table 1), and then were separated by electrophoresis for 1 h at 100 V in a 1.5% agarose gel. Random samples (10% of the total samples) were analyzed by DNA sequencing methods with an ABI 3730 sequencer (Bioasia Biotechnology Co., Ltd. Shanghai, China) to verify the genotype results of allelic variation, which were based on the electrophoretic pattern of the restriction enzyme-treated PCR products.

Statistical analyses

Allele and genotype frequencies were calculated in the studied subjects. The chi-squared test (χ^2) was used to assess the Hardy-Weinberg equilibrium of the genotypes of different genetic mutations. The BMD values were adjusted by age, height, and weight through stepwise multiple regression and logistic regression analyses. The one-way analysis of variance (ANOVA) and least-significant difference (LSD) post hoc test were utilized to compare the quantitative data. All statistical analyses were performed with the Statistical Package for Social Sciences software (SPSS, Windows version release 15.0; SPSS Inc.; Chicago, IL, USA). All data were presented as the mean ± SD (standard deviation of the mean). A P value of <0.05 was considered statistically significant.

RESULTS

SNPs genotyping

In the present study, two novel genetic variants (g.19163A>G and g.23298T>C) were detected by PCR-RFLP, CRS-PCR, and DNA sequencing methods, including A→G mutation (resulting in phenylalanine (Phe) to leucine (Leu) amino acid replacement, p.Phe117Leu) at position 19163 and T→C mutation (not resulting in amino acid replacement, p.Arginine (Arg)161Arg) at position 23298 of the human *OPG* gene, respectively in exon2 and exon3 (reference sequences, GenBank IDs: NG_012202.1, NM_002546.3 and NP_002537.3). The PCR amplified products of g.19163A>G was digested with *Sac*I restriction enzyme and divided into three genotypes: AA (256 bp), AG (256, 183 and 73 bp) and GG (183 and 73 bp) (Table 1). The PCR amplified products of g.23298T>C was digested with the *Taq*I restriction enzyme and divided into three genotypes: TT (190 and 20 bp), TC (210, 190 and 20 bp), and CC (210 bp) (Table 1).

Genotype and allele frequencies

The result of the χ^2 test for g.19163A>G and g.23298T>C variants in the studied populations indicated that these genetic variants were in the

Table 2. Genotypic and allelic frequencies of g.19163A>G and g.23298T>C polymorphisms in the studied subjects

Groups	g.19163A>G					g.23298T>C				
	Genotype frequencies (%)			Allele frequencies (%)		Genotype frequencies (%)			Allele frequencies (%)	
	AA	AG	GG	A	G	TT	TC	CC	T	C
Case group (n=367)	121 (32.97)	166 (45.23)	80 (21.80)	408 (55.59)	326 (44.41)	191 (52.04)	141 (38.42)	35 (9.54)	523 (71.25)	211 (28.75)
Control group (n=372)	161 (43.28)	159 (42.74)	52 (13.98)	481 (64.65)	263 (35.35)	201 (54.03)	151 (40.59)	20 (5.38)	553 (74.33)	191 (25.67)
Total (n=739)	282 (38.16)	325 (43.98)	132 (17.86)	889 (60.15)	589 (39.85)	392 (53.05)	292 (39.51)	55 (7.44)	1076 (72.80)	402 (27.20)
	P = 0.003[a]			P < 0.001[a]		P = 0.098[a]			P = 0.184[a]	

Note: [a]P-value was evaluated by χ^2 test.

Hardy-Weinberg equilibrium (P >0.05). Table 2 presents the genotype and allele frequencies of the g.19163A>G and g.23298T>C variants. As for the g.19163A>G variant, the allele frequencies of the cases (A: 55.59% and G: 44.41%) were significantly different from those of the controls (A: 64.65% and G: 35.35%, P < 0.001, χ^2 test, Table 2). Additionally, the genotype frequencies in cases were not similar to those of controls, the differences being statistically significant (P = 0.003, χ^2 test, Table 2). As for the g.23298T>C variant, the allele and genotype frequencies of cases (for allele, T: 71.25% and C: 28.75%; for genotype, TT: 52.04%, TC: 38.42%, and CC: 9.54%) showed differences from those of the controls (for allele, T: 74.33% and C: 25.67%; for genotype, TT: 54.03%, TC: 40.59%, and CC: 5.38%), the differences, however, not being statistically significant (for allele, P = 0.184; for genotype, P = 0.098, χ^2 test, Table 2).

Association between OPG genetic variants and bone mineral density

Age, height, weight, body mass index (BMI), spine BMD, neck hip BMD, and total hip BMD in each genotype group were presented as the mean ± SD (Table 3). The results of this study suggest that g.19163A>G SNP is significantly associated with adjusted spine BMD, neck hip BMD, and total hip BMD. Subjects with genotype AA had significantly higher BMD value than those of genotypes AG and GG (P <0.05, Table 3). However, we failed to detect any significant association between g.23298T>C SNP and adjusted spine BMD, neck hip BMD, and total hip BMD in the present study (P >0.05, Table 3).

DISCUSSION

The *OPG* gene is an important candidate gene in the pathogenesis of osteoporosis.[14-22] Several studies have reported the association between genetic variants and osteoporosis and BMD.[6,7,15,19,22,23,34-39] However, the results from these observations still remain poorly understood. In this study, we firstly detected the association of g.19163A>G and g.23298T>C SNPs of the *OPG* gene with osteoporosis and BMD. Our data indicated that g.19163A>G was significantly associated with osteoporosis and BMD. Allele-A could be a risk factor for osteoporosis in Chinese postmenopausal women. As for g.23298T>C, we failed to detect any statistically significant association between this variant and adjusted spine BMD, neck hip BMD, and total hip BMD (P >0.05). It seems that the allele-C might have potential effects on osteoporosis and BMD. These findings suggest that the *OPG* gene is related to osteoporosis and BMD in Chinese postmenopausal women. Previously published studies have demonstrated the association between

Table 3. Characteristics of g.19163A>G and g.23298T>C polymorphisms in the total group of subjects

SNPs	g.19163A>G				g.23298T>C			
Genotype	AA	AG	GG	*P	TT	TC	CC	*P
Number (%)	282 (38.16)	325 (43.98)	132 (17.86)	-	392 (53.05)	292 (39.51)	55 (7.44)	-
Age (years)	61.6±6.9	62.3±7.4	62.8±7.2	0.543a	61.5±6.8	62.6±7.7	62.8±7.2	0.443[a]
Height (cm)	160±7.1	162±6.5	163±6.8	0.432a	159±7.1	161±6.6	163±6.9	0.333[a]
Weight (kg)	60.4±7.4	62.5±6.2	62.8±5.8	0.351a	61.3±7.6	63.2±6.7	63.5±6.7	0.431[a]
BMI	23.1±3.33	23.2±3.45	23.6±3.29	0.118b	23.2±3.36	23.3±3.41	23.8±3.36	0.256[a]
Adjusted spine BMD (g/cm²)[c]	0.926±0.113	0.852±0.122	0.835±0.121	0.026b	0.864±0.126	0.832±0.135	0.829±0.121	0.125[b]
Adjusted Neck hip BMD (g/cm²)[c]	0.740±0.118	0.688±0.123	0.679±0.137	0.029b	0.719±0.108	0.695±0.111	0.689±0.125	0.321[b]
Adjusted Total hip BMD (g/cm²)[c]	0.859±0.110	0.810±0.105	0.806±0.125	0.042b	0.841±0.217	0.831±0.128	0.828±0.122	0.052[b]

Note: SNPs mean single-nucleotide polymorphisms; BMI means body mass index; BMD means bone mineral density; Data are shown as mean ± SD. [a] P-values were evaluated by one-way analysis of variance (ANOVA) test; [b] P-values were evaluated by one-way analysis of variance (ANOVA) and least-significant difference (LSD) post hoc test; [c] BMD values were adjusted by age, height and weight through stepwise multiple regression and logistic regression analyses).

SNPs (e.g., A163G, T245G, T950C and G1181C) osteoporosis and BMD in different ethnicities, which is consistent with our findings, and that genetic variants in the *OPG* gene may contribute to genetic effects on osteoporosis and BMD.[6,7,15,19,22,23,34-39] Further studies in larger populations to investigate the association of g.19163A>G, g.23298T>C, or other genetic variants, with osteoporosis and BMD are necessary to verify these results and to further elucidate the pathogenesis and molecular mechanism of osteoporosis.

In conclusion, our data suggest that the *OPG* g.19163A>G variant could be associated with osteoporosis and BMD. The results of this study could form the basis for further analysis of the implication of *OPG* genetic variants in osteoporosis risk factors.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES

1. Cummings SR, Kelsey JL, Nevitt MC, O'Dowd KJ, 1985 Epidemiology of osteoporosis and osteoporotic fractures. Epidemiol Rev 7: 178-208.
2. Riggs BL, Melton LJ 3rd, 1986 Involutional osteoporosis. N Engl J Med 314: 1676-1686.
3. Kanis JA, Melton LJ 3rd, Christiansen C, Johnston CC, Khaltaev N 1994 The diagnosis of osteoporosis. J Bone Miner Res 9: 1137-1141.
4. Geng L, Yao Z, Yang H, Luo J, Han L, et al, 2007 Association of CA repeat polymorphism in estrogen receptor beta gene with postmenopausal osteoporosis in Chinese. J Genet Genomics 34: 868-876.
5. Peck WA, 1993 Consensus development conference: diagnosis, prophylaxis, and treatment of osteoporosis. Am J Med 94: 646-650.
6. Garcia-Unzueta MT, Riancho JA, Zarrabeitia MT, et al, 2008 Association of the 163A/G and 1181G/C osteoprotegerin polymorphism with bone mineral density. Horm Metab Res 40: 219-224.
7. Ohmori H, Makita Y, Funamizu M, et al, 2002 Linkage and association analyses of the osteoprotegerin gene locus with human osteoporosis. J Hum Genet 47: 400-406.
8. Albagha OM, Ralston SH, 2006 Genetics and osteoporosis. Rheum Dis Clin North Am 32: 659-680.
9. Hosoi T, 2010 Genetic aspects of osteoporosis. J Bone Miner Metab 28: 601-607.
10. Ralston SH, 2010 Genetics of osteoporosis. Ann N Y Acad Sci 1192: 181-189.
11. Ferrari S, 2008 Human genetics of osteoporosis. Best Pract Res Clin Endocrinol Metab 22: 723-735.
12. Cheung CL, Xiao SM, Kung AW, 2010 Genetic epidemiology of age-related osteoporosis and its clinical applications. Nat Rev Rheumatol 6: 507-517.
13. Woo J, Kim Y, Lee C, 2012 Heterogeneous genetic associations of nucleotide sequence variants with bone mineral density by gender. Mol Biol Rep 39: 2259-2265.
14. Pocock NA, Eisman JA, Hopper JL, Yeates MG, Sambrook PN, Eberl S, 1987 Genetic determinants of bone mass in adults. A twin study. J Clin Invest 80: 706-710.
15. Langdahl BL, Carstens M, Stenkjaer L, Eriksen EF, 2002 Polymorphisms in the osteoprotegerin gene are associated with osteoporotic fractures. J Bone Miner Res 17: 1245-1255.
16. Arko B, Prezelj J, Kocijancic A, Komel R, Marc J, 2005 Association of the osteoprotegerin gene polymorphisms with bone mineral density in postmenopausal women. Maturitas 51: 270-279.
17. Yamada Y, Ando F, Niino N, Shimokata H, 2003 Association of polymorphisms of the osteoprotegerin gene with bone mineral density in Japanese women but not men. Mol Genet Metab 80: 344-349.
18. Vidal C, Formosa R, Xuereb-Anastasi A, 2011 Functional polymorphisms within the TNFRSF11B (osteoprotegerin) gene increase the risk for low bone mineral density. J Mol Endocrinol 47: 327-333.
19. Arko B, Prezelj J, Komel R, Kocijancic A, Hudler P, Marc J, 2002 Sequence variations in the osteoprotegerin gene promoter in patients with postmenopausal osteoporosis. J Clin Endocrinol Metab 87: 4080-4084.
20. Hofbauer LC, Schoppet M, 2002 Osteoprotegerin gene polymorphism and the risk of osteoporosis and vascular disease. J Clin Endocrinol Metab 87: 4078-4079.
21. Hussien YM, Shehata A, Karam RA, Alzahrani SS, Magdy H, el-Shafey, 2012 Polymorphism in vitamin D receptor and osteoprotegerin genes in Egyptian rheumatoid arthritis patients with and without osteoporosis. Mol Biol Rep 40: 3675-3680.
22. Jurado S, Nogues X, Agueda L, et al, 2010 Polymorphisms and haplotypes across the osteoprotegerin gene associated with bone mineral density and osteoporotic fractures. Osteoporos Int 21: 287-296.
23. Paternoster L, Ohlsson C, Sayers A, et al, 2010 OPG and RANK polymorphisms are both associated with cortical bone mineral density: findings from a metaanalysis of the Avon longitudinal study of parents and children and gothenburg osteoporosis and obesity determinants cohorts. J Clin Endocrinol Metab 95: 3940-3948.
24. Fang Y, van Meurs JB, d'Alesio A, et al, 2005 Promoter and 3'-untranslated-region haplotypes in the vitamin d receptor gene predispose to osteoporotic fracture: the rotterdam study. Am J Hum Genet 77: 807-823.
25. Li Y, Xi B, Li K, Wang C, 2012 Association between vitamin D receptor gene polymorphisms and bone mineral density in Chinese women. Mol Biol Rep 39:

5709-5717.

26. Kurt O, Yilmaz-Aydogan H, Uyar M, Isbir T, Seyhan MF, Can A, 2012 Evaluation of ERalpha and VDR gene polymorphisms in relation to bone mineral density in Turkish postmenopausal women. Mol Biol Rep 39: 6723-6730.

27. Jakubowska-Pietkiewicz E, Mlynarski W, Klich I, Fendler W, Chlebna-Sokol D, 2012 Vitamin D receptor gene variability as a factor influencing bone mineral density in pediatric patients. Mol Biol Rep 39: 6243-6250.

28. Horst-Sikorska W, Dytfeld J, Wawrzyniak A, et al, 2013 Vitamin D receptor gene polymorphisms, bone mineral density and fractures in postmenopausal women with osteoporosis. Mol Biol Rep 40: 383-390.

29. Gennari L, Merlotti D, De Paola V, et al, 2005 Estrogen receptor gene polymorphisms and the genetics of osteoporosis: a HuGE review. Am J Epidemiol 161: 307-320.

30. Ioannidis JP, Ralston SH, Bennett ST, et al, 2004 Differential genetic effects of ESR1 gene polymorphisms on osteoporosis outcomes. JAMA 292: 2105-2114.

31. Ioannidis JP, Stavrou I, Trikalinos TA, et al, 2002 Association of polymorphisms of the estrogen receptor alpha gene with bone mineral density and fracture risk in women: a meta-analysis. J Bone Miner Res 17: 2048-2060.

32. Mann V, Ralston SH, 2003 Meta-analysis of COL1A1 Sp1 polymorphism in relation to bone mineral density and osteoporotic fracture. Bone 32: 711-717.

33. Yamada Y, 2001 Association of polymorphisms of the transforming growth factor-beta1 gene with genetic susceptibility to osteoporosis. Pharmacogenetics 11: 765-771.

34. Zhao HY, Liu JM, Ning G, et al, 2005 The influence of Lys3Asn polymorphism in the osteoprotegerin gene on bone mineral density in Chinese postmenopausal women. Osteoporos Int 16: 1519-1524.

35. Lee YH, Woo JH, Choi SJ, Ji JD, Song GG, 2010 Associations between osteoprotegerin polymorphisms and bone mineral density: a meta-analysis. Mol Biol Rep 37: 227-234.

36. Moffett SP, Oakley JI, Cauley JA, et al, 2008 Osteoprotegerin Lys3Asn polymorphism and the risk of fracture in older women. J Clin Endocrinol Metab 93: 2002-2008.

37. Kim JG, Kim JH, Kim JY, et al, 2007 Association between osteoprotegerin (OPG), receptor activator of nuclear factor-kappaB (RANK), and RANK ligand (RANKL) gene polymorphisms and circulating OPG, soluble RANKL levels, and bone mineral density in Korean postmenopausal women. Menopause 14: 913-918.

38. Ueland T, Bollerslev J, Wilson SG, et al, 2007 No associations between OPG gene polymorphisms or serum levels and measures of osteoporosis in elderly Australian women. Bone 40: 175-181.

39. Jorgensen HL, Kusk P, Madsen B, Fenger M, Lauritzen JB, 2004 Serum osteoprotegerin (OPG) and the A163G polymorphism in the OPG promoter region are related to peripheral measures of bone mass and fracture odds ratios. J Bone Miner Metab 22: 132-138.

40. Haliassos A, Chomel JC, Tesson L, et al, 1989 Modification of enzymatically amplified DNA for the detection of point mutations. Nucleic Acids Res 17: 3606.

41. Yuan ZR, Li J, Li JY, Gao X, Xu SZ, 2013 SNPs identification and its correlation analysis with milk somatic cell score in bovine MBL1 gene. Mol Biol Rep 40: 7-12.

42. Yuan ZR, Li JY, Li J, et al, 2012 Investigation on BRCA1 SNPs and its effects on mastitis in Chinese commercial cattle. Gene 505: 190-194.

43. Yuan ZR, Li JY, Li J, Gao X, Xu SZ, 2013 Effects of DGAT1 gene on meat and carcass fatness quality in Chinese commercial cattle. Mol Biol Rep 40: 1947-1954.

44. Zhao CJ, Li N, Deng XM, 2003 The establishment of method for identifying SNP genotype by CRS-PCR. Yi Chuan 25: 327-329.