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ORIGINAL ARTICLE

The association of osteoprotegerin gene polymorphisms with periodontitis

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BACKGROUND: It has been demonstrated that genetic variation accounts for approximately half of the variance in periodontitis. The reported association of polymorphisms in the osteoprotegerin (OPG) gene with osteoporosis suggests that the OPG gene may also influence the genetic risk for periodontitis.

SUBJECTS AND METHODS: We investigated the distribution of OPG gene polymorphisms in 49 patients with aggressive (*n* = 14) or chronic (*n* = 35) periodontitis and 49 control subjects without periodontitis, using polymerase chain reaction (PCR)–restriction fragment length polymorphism and PCR–single strand conformation polymorphism followed by direct sequencing.

RESULTS: A total of seven known polymorphisms and one new mutation, G373A, were identified. The T950 and G1181 alleles were more common in patients with periodontitis (*P* = 0.028 and *P* = 0.047, respectively) than in control subjects. Especially, G1181 allele was associated with patients with aggressive periodontitis.

CONCLUSION: The TG haplotype of T950C and G1181C polymorphisms in the OPG gene may be useful genetic markers for the prediction of periodontitis. Further studies in a larger population are required to determine whether these alleles directly contribute to periodontitis susceptibility.

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Keywords: osteoprotegerin; polymorphism; periodontitis; risk factor

Introduction

The pathogenesis of periodontitis involves a complex interplay of microbial and environmental factors with the genetic factors that determine the host immune response to bacteria. It has been demonstrated that approximately half of the variance in periodontitis is attributed to genetics (Michalowicz *et al*, 2000). Many groups have studied the association between periodontitis and polymorphisms of candidate genes, including pro- and anti-inflammatory cytokines, innate immune recognition receptors, Fcγ receptors, and the vitamin D receptor (VDR). Although the association of periodontitis with specific genotypes of interleukin-1, TNF-α, Fcγ receptors, and VDR has been reported (Kinane *et al*, 2005), no single gene is sufficient or essential for the development of periodontitis. Therefore, like many other common human diseases, periodontitis appears to be influenced by more than one gene, and the identification of such genes would provide a valuable tool for risk assessment.

The inflammatory response to bacteria present in subgingival plaque leads to the destruction of alveolar bones, a hallmark of periodontitis. Bone mass in adults is the result of continuous remodeling processes that involve resorption by osteoclasts and new bone formation by osteoblasts (Brandstrom *et al*, 2001). Osteoblasts regulate the differentiation and activity of osteoclasts through the expression of receptor activator NF-κB ligand (RANKL) and osteoprotegerin (OPG) (Roux and Orcel, 2000). Together with M-CSF, RANKL is a key cytokine in the induction of osteoclastogenesis both *in vitro* and *in vivo* (Suda *et al*, 1999). OPG, a soluble decoy receptor secreted by osteoblasts, binds RANKL and inhibits osteoclastogenesis (Brandstrom *et al*, 2001; Wada *et al*, 2001). The ratio of RANKL to OPG in the microenvironment determines the balance between bone formation and resorption (Bucay *et al*, 1998; Roux and Orcel, 2000; Brandstrom *et al*, 2001). Mogi *et al* (2004) reported that patients with periodontitis have a significantly higher ratio of RANKL to OPG in gingival crevicular fluid, suggesting a role for RANKL and OPG in alveolar bone destruction. This, in addition to the reported association of OPG gene polymorphisms with other diseases associated with increased osteoclast bone resorption,

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suggests that the OPG gene may be one of the genetic risk factors for periodontitis.

In this study, we investigated the distribution of OPG gene polymorphisms in patients with periodontitis and in control individuals and showed that the T950 and G1181 alleles are associated with periodontitis in a Korean population.

Materials and methods

Subjects

The subjects for this study were 49 healthy control subjects, 14 patients with aggressive periodontitis, and 35 with chronic periodontitis who visited the Department of Periodontology at Seoul National University Dental Hospital (Table 1). We followed the ethical principles for medical research involving human subjects of the World Medical Association declaration of Helsinki. The study was approved by the Institute Review Board at Seoul National University and written informed consent was obtained from all subjects. All subjects were Korean and had no systemic disease. Clinical parameters, including probing depth (PD), clinical attachment level (CAL), bleeding on probing, gingival index, and plaque index were assessed for each subject. PD and CAL were recorded using the Florida Probe® (Florida Probe Co., Gainesville, FL, USA) in six sites for each tooth. Control subjects must have neither CAL nor PD >4 mm in more than one site. Clinical criteria for aggressive periodontitis included the following: ≥ 5 mm CAL at more than one site on ≥ 8 teeth, at least three of which were not first molars and incisors, and disease onset before the age of 35 years.

Table 1 Subjects for OPG polymorphisms

	Control	*Aggressive periodontitis*	*Chronic periodontitis*
Gender			
Male	35	9	14
Female	14	5	21
Mean age (years)	27	35	48
%PI	43.26	74.67	70.32
Mean PPD (mm)	2.07	3.72	3.31
Mean CAL (mm)	2.10	4.38	3.84
%BOP	10.59	57.80	45.63
Mean GI	0.20	1.03	0.84
Current smoker (%)	18	21	28

%PI, supragingival plaque accumulation; PPD, probing pocket depth; CAL, clinical attachment level; %BOP, % bleeding on probing; GI, gingival index; OPG, osteoprotegerin.

DNA

Genomic DNA was prepared from peripheral blood using the Puregene blood kit (Gentra Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Polymerase chain reaction

Polymerase chain reaction was performed in a total volume of 30 μl containing 1X PCR buffer, 1.25U Taq polymerase (TaKaRa Shuzo, Shiga, Japan), 0.2 μM dNTPs, 0.3 μM each primer (Table 2), and 100 ng DNA. Amplifications were performed in a thermal cycler (GeneAmp PCR system 9600; Perkin-Elmer, Norwalk, CT, USA) with 35 cycles of denaturation at 96°C for 45 s, annealing at the temperatures denoted in the supplementary table for 45 s, and extension at 72°C for 45 s, with an initial denaturation at 96°C for 5 min and a final extension at 72°C for 10 min.

Restriction fragment length polymorphisms

Polymerase chain reaction products containing the A163G, T245G, and T950C polymorphisms in the promoter region of the OPG gene were digested with 8 U of *Hin*fI (TaKaRa Shuzo), *Ase*I, and *Hin*cII (New England Biolabs, Hitchin, UK), respectively, at 37°C for 2 h. Digestion products were separated on ethidium

Table 2 Primer sequences amplifying osteoprotegerin gene

Region	*Usage*[a]	*Primer sequence (5′ → 3′)*	*Annealing temperature*
Promoter			
A163G/T245G	G/S	Forward-AACTTGAACACTTGG CCCTGA Reverse-AAATTGGACTGCCTGGGG	60
T950C	G/S	Forward-GTTCCTCAGCCCGGTGGCTTTT Reverse-TGTGGTCCCCGGAAACTTCAGG	64
Exon			
1	G	Forward-TCGCCCAGCCGCCGCCTCC Reverse-GGGCACCGTCGGCTGG	60
	S	Forward-ACCACCGCCCCACCCCTCAC Reverse-TCCAGCCTAACCCCAAGCCTCTCC	66
2	G/S	Forward-TTTCATGCTAAGATGATGCC Reverse-ATCCTAATTAATTTTGCTGCAC	57
3	G/S	Forward-AAACGATTTGAGGAGAAGG Reverse-AGAGATGATACTACAAAATCG	50
4	G/S	Forward-AATTGTTGAGTAAATCTTCTGGG Reverse-TGGTTATGATAAATAGGTGTC	55
5	G/S	Forward-AATGATGTGAACACTTATCTGG Reverse-TGAGGAAACAGCTCAATGGC	55

[a]G, genotyping; S, sequencing.

bromide-stained agarose gels (3.0%) and visualized using a LAS-1000 Plus luminescent image analyzer (Fuji, Tokyo, Japan).

Single strand conformation polymorphism

All exons and intron–exon boundaries of the OPG gene were screened for polymorphisms or mutations using PCR–single strand conformation polymorphism (SSCP). Five microliter PCR product was mixed with 10 μl SSCP loading buffer (95% formamide, 1 M NaOH, 0.05% bromophenol blue, and 0.05% xylene cyanol), and denatured at 95°C for 5 min followed by rapid chilling on ice for at least 5 min. Samples were separated through a non-denaturing 8% polyacrylamide gel (19:1 acrylamide:bisacrylamide) at 20 W for 18 h at 4°C. After electrophoresis, gels were stained with SYBER Green I (Roche, Mannheim, Germany) and visualized by a LAS-1000 Plus luminescent image analyzer (Fuji). For exons 2 and 5, the PCR products were digested with *Kpn*I (TaKaRa Shuzo) and *Hin*dIII (TaKaRa Shuzo), respectively, before undergoing SSCP gel analysis.

Sequencing of PCR products

Polymerase chain reaction products were purified with the QIAquick PCR purification kit (QIAGEN GmbH, Hilden, Germany), and sequenced at TaKaRa Korea Biomedical Inc., Seoul, Korea.

Statistical analysis

Differences in the prevalence of genotypes and alleles between patients with periodontitis and control subjects were analyzed using the Pearson's chi-squared test and Fisher's exact test. $P < 0.05$ was considered statistically significant. Odds ratios (OR) were adjusted for smoking using logistic regression analysis. Statistical analysis was performed with the SPSS version 12.0 K for Windows (SPSS Inc., Chicago, IL, USA). ISTECH SNPAnalyzer (ISTECH Inc., Goyangsi, Korea) was used to test for haplotype.

Results

During PCR–SSCP analysis of the 98 individuals, three different migration patterns were observed for exon 1, and two patterns were observed for exons 2, 3, and 4 (Figure 1). No variation in migration was observed for exon 5. Direct sequencing of the PCR products revealed that these band patterns were caused by the known polymorphisms G1181C, C4441T, and A6833G/A6890G in exon 1, exon 3, and exon 4, respectively. The migration pattern observed in exon 2 was caused by the G373A allele, which results in the amino acid change, Arg131His. Only one control individual out of 98 subjects carried the A373 variant in one allele, suggesting that it may be a novel mutation.

The distributions of genotypes and allele frequencies in patients with periodontitis and controls are summarized in Table 3. The A6833G allele was completely linked with A6890G, as reported previously (Langdahl *et al*, 2002), but T245G was not completely linked with C4441T in our study population. Although the difference in genotype distribution did not reach statistical significance, the T950 allele was significantly over-represented in patients with periodontitis, compared to normal controls ($P = 0.028$). The G1181 allele was also more common among patients with periodontitis ($P = 0.047$), especially in those with an aggressive type of diseases ($P = 0.012$). The ORs for periodontitis in individuals with the T950 allele and G1181 allele were 0.60 (95% CI: 0.239–1.503) and 0.55 (95% CI: 0.214–1.396), respectively; these values were not statistically significant. A significant OR of 0.22 (95% CI: 0.054–0.898) was only obtained when the G1181 allele in patients with aggressive periodontitis. The T950C polymorphism was not associated with periodontitis and the G1181C polymorphism was associated with patients with aggressive periodontitis.

Haplotype analysis of T950G-G1181C polymorphisms revealed that most of the subjects in our population had the TG haplotype. Differences in the distribution of the haplotypes between control and patients with periodontitis reached significance ($\chi^2 = 10.16$, d.f. = 3, and $P = 0.017$) (Table 4) with the TG haplotype being more frequent in patients. The CC haplotypes were more prevalent in control than in patients; this haplotype seems to protective effect.

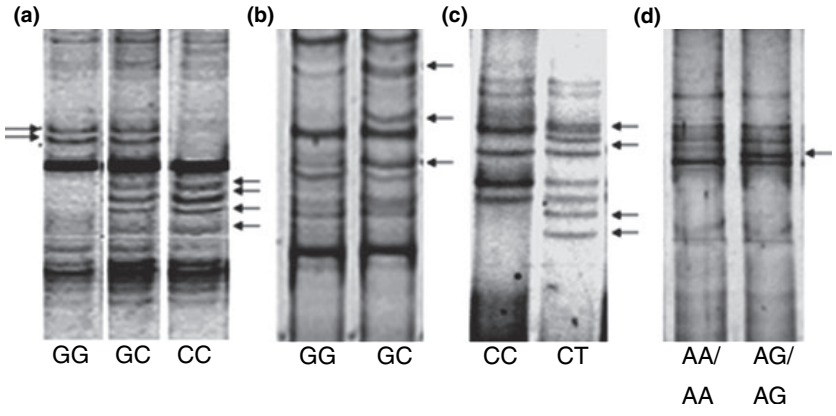

Figure 1 Different migration patterns analyzed by polymerase chain reaction–single strand conformation polymorphism and their corresponding genotypes. (**a**), G1181C in exon 1: lane 1 wild type carrier (GG); lane 2 heterozygous carrier (GC); lane 3 homozygous variant carrier (CC). (**b**), G373A in exon 2: lane 1 wild type carrier (GG); lane 2 heterozygous carrier (GC). (**c**), C4441T: lane 1 wild type carrier (CC); lane 2 heterozygous carrier (CT). (**d**), A6833G/A6890G: lane 1 wild type carrier (AA/AA); lane 2 heterozygous carrier (AG/AG)

Table 3 Genotype frequencies and allele frequencies of the osteoprotegerin gene polymorphisms

		Genotype (% frequency)					*Allele (% frequency)*			
	Group	*11*	*12*	*22*	χ^2	*P*-value[a]	*1*	*2*	*P*-value[b]	*OR (95% CI)*
A163G	Control	28 (57)	21 (43)	0			77 (79)	21 (21)	0.721	
	Periodontitis	32 (66)	16 (33)	1 (1)	1.942	0.379	80 (82)	18 (18)	1	
	Aggresssive P	8 (57)	6 (43)	0	0	1	22 (79)	6 (21)	0.558	
	Chronic P	24 (69)	10 (29)	1 (2)	2.96	0.227	58 (83)	12 (17)		
T245G	Control	30 (61)	19 (39)	0			79 (81)	19 (19)	1.0	
	Periodontitis	31 (61)	18 (37)	0	0.043	0.835	80 (83)	18 (17)	0.793	
	Aggresssive P	8 (57)	6 (43)	0	0.076	0.783	22 (79)	6 (21)	0.841	
	Chronic P	23 (66)	12 (34)	0	0.177	0.673	58 (83)	12 (17)		
T950C	Control	15 (31)	21 (43)	13 (26)			51 (52)	47 (48)		
	Periodontitis	23 (47)	21 (43)	5 (10)	5.240	0.073	67 (68)	31 (32)	0.028*	0.60 (0.23–1.50)
	Aggresssive P	7 (50)	6 (43)	1 (7)	3.010	0.220	20 (71)	8 (29)	0.085	
	Chronic P	16 (46)	15 (43)	4 (11)	3.563	0.168	47 (67)	23 (33)	0.058	
G1181C	Control	21 (43)	17 (35)	11 (22)			59 (60)	39 (40)		
	Periodontitis	31 (63)	11 (22)	7 (15)	4.098	0.128	73 (75)	25 (25)	0.047*	0.55 (0.21–1.39)
	Aggresssive P	11 (79)	2 (14)	1 (7)	5.577	0.061	24 (86)	4 (14)	0.013*	0.22 (0.05–0.89)*
	Chronic P	20 (57)	9 (26)	6 (17)	1.670	0.434	49 (70)	21 (30)	0.253	
C4441T	Control	34 (69)	15 (31)	0			83 (85)	15 (15)		
	Periodontitis	31 (63)	18 (37)	0	0.411	0.521	80 (82)	18 (18)	0.703	
	Aggresssive P	8 (57)	6 (43)	0	0.735	0.391	22 (79)	6 (21)	0.565	
	Chronic P	23 (66)	12 (34)	0	0.126	0.722	58 (83)	12 (17)	0.832	
A6833G/ A6890G	Control	43 (98)	1 (2)	0			97 (99)	1 (1)		
	Periodontitis	47 (97)	2 (3)	0	0.344	0.557	96 (98)	2 (2)	1.0	
	Aggresssive P	14 (100)	0	0	0.290	0.590	28 (100)	0	1.0	
	Chronic P	33 (94)	2 (6)	0	0.800	0.765	68 (97)	2 (3)	0.571	

[a]Statistical difference of genotype using a Pearsons chi-squared test. [b]Statistical difference of allele frequency using a Fisher's exact test: control *vs* periodontitis (aggressive periodontitis + chronic periodontitis); control *vs* aggressive periodontitis; control *vs* chronic periodontitis. *$P < 0.05$. OR, odds ratios; Cl, confidence interval, OR was adjusted for smoking.

Table 4 Haplotypes of T95OG and G1811C polymorphism

Haplotype sequence		*Patients*		*Control*	
T950C	*G1181C*	*Number*	*Frequency*	*Number*	*Frequency*
T	G	59	0.605	44	0.464
C	G	16	0.169	14	0.150
C	C	14	0.146	32	0.339
T	C	7	0.078	4	0.046

Comparisons of haplotype frequencies were performed by chi-squared test. Haplotypes were distributed significantly different in patients *vs* control ($X^2 = 10.16$, d.f. = 3, and $P = 0.017$).

Discussion

In this study, we investigated the association of OPG gene polymorphisms with periodontitis and demonstrated that the T950 and G1181 alleles are more common in patients with periodontitis than in control subjects. We found no association between periodontitis and the T245G polymorphism, consistent with the findings of Wohlfahrt *et al* (2006), who previously reported no association between the T245G polymorphism and severe chronic periodontitis. We describe a new allelic variant, G373A, that results in the amino acid change Arg131His. The carrier of this variant was a 28-year-old male who was healthy both periodontally and systemically. Further clinical follow-up is needed to assess the functional consequence of this variation and its role in periodontitis.

Many groups have studied the association of OPG gene polymorphisms with osteoporosis and other diseases involving osteolysis or calcification. The association of the G1181 allele with osteoporosis or low bone mineral density (BMD) has been reported most often in diverse ethnic groups including Korean populations (Arko *et al*, 2002; Langdahl *et al*, 2002; Choi *et al*, 2005; Zhao *et al*, 2005; Vidal *et al*, 2006). The G1181 allele has been found to increase the risk of developing Paget's disease (Daroszewska *et al*, 2004). An association between the T950 allele and osteoporosis or low BMD has been reported by two groups (Ohmori *et al*, 2002; Vidal *et al*, 2006), although other groups have reported no association or even opposite results (Langdahl *et al*, 2002; Wynne *et al*, 2002; Yamada *et al*, 2003; Brandstrom *et al*, 2004). In contrast, the C950 allele was positively correlated with serum OPG levels and an increased risk of coronary artery disease (Brandstrom *et al*, 2002; Soufi *et al*, 2004). Although there is some inconsistency in the literature, the T950 and G1181 alleles seem to be associated with increased osteoclast function. The association between osteoporosis and periodontitis has been recognized by many groups, and postmenopausal osteopenia has been implicated as a risk indicator for periodontitis (Tezal *et al*, 2000; Wactawski-Wende, 2001; Reddy, 2002). Because these two highly prevalent diseases are commonly associated with OPG polymorphisms, severe periodontitis in middle age

could be a risk indicator for osteoporosis with advancing age.

Despite the small study population, we observed significant differences in the distribution of specific polymorphisms in the OPG gene between periodontitis patients and normal individuals. Although the functional basis is not clear, TG haplotype of T950C and G1181C polymorphisms was associated with periodontitis, suggesting that these could be useful genetic markers in predicting periodontitis. In light of the importance of the OPG gene in periodontitis, future studies are planned to expand this investigation to a larger population.

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References

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.

Brandstrom H, Bjorkman T, Ljunggren O (2001). Regulation of osteoprotegerin secretion from primary cultures of human bone marrow stromal cells. *Biochem Biophys Res Commun* **280:** 831–835.

Brandstrom H, Stiger F, Lind L, Kahan T, Melhus H, Kindmark A (2002). A single nucleotide polymorphism in the promoter region of the human gene for osteoprotegerin is related to vascular morphology and function. *Biochem Biophys Res Commun* **293:** 13–17.

Brandstrom H, Gerdhem P, Stiger F *et al* (2004). Single nucleotide polymorphisms in the human gene for osteoprotegerin are not related to bone mineral density or fracture in elderly women. *Calcif Tissue Int* **74:** 18–24.

Bucay N, Sarosi I, Dunstan CR. *et al* (1998). Osteoprotegerin-deficient mice develop early onset osteoporosis and arterial calcification. *Genes Dev* **12:** 1260–1268.

Choi JY, Shin A, Park SK *et al* (2005). Genetic polymorphisms of OPG, RANK, and ESR1 and bone mineral density in Korean postmenopausal women. *Calcif Tissue Int*. **77:** 152–159.

Daroszewska A, Hocking LJ, McGuigan FE *et al* (2004). Susceptibility to Paget's disease of bone is influenced by a common polymorphic variant of osteoprotegerin. *J Bone Miner Res* **19:** 1506–1511.

Kinane DF, Shiba H, Hart TC (2005). The genetic basis of periodontitis. *Periodontol 2000* **39:** 91–117.

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.

Michalowicz BS, Diehl SR, Gunsolley JC *et al* (2000). Evidence of a substantial genetic basis for risk of adult periodontitis. *J Periodontol* **71:** 1699–1707.

Mogi M, Otogoto J, Ota N, Togari A (2004). Differential expression of RANKL and osteoprotegerin in gingival crevicular fluid of patients with periodontitis. *J Dent Res* **83:** 166–169.

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.

Reddy MS (2002). Oral osteoporosis: is there an association between periodontitis and osteoporosis? *Compend Contin Educ Dent* **23:** 21–28.

Roux S, Orcel P (2000). Bone loss. Factors that regulate osteoclast differentiation: an update. *Arthritis Res* **2:** 451–456.

Soufi M, Schoppet M, Sattler AM *et al* (2004). Osteoprotegerin gene polymorphisms in men with coronary artery disease. *J Clin Endocrinol Metab* **89:** 3764–3768.

Suda T, Takahashi N, Udagawa N, Jimi E, Gillespie MT, Martin TJ (1999). Modulation of osteoclast differentiation and function by the new members of the tumor necrosis factor receptor and ligand families. *Endocr Rev*. **20:** 345–357.

Tezal M, Wactawski-Wende J, Grossi SG, Ho AW, Dunford R, Genco RJ (2000). The relationship between bone mineral density and periodontitis in postmenopausal women. *J Periodontol* **71:** 1492–1498.

Vidal C, Brincat M, Xuereb Anastasi A (2006). TNFRSF11B gene variants and bone mineral density in postmenopausal women in Malta. *Maturitas* **53:** 386–395.

Wactawski-Wende J (2001). Periodontal diseases and osteoporosis: association and mechanisms. *Ann Periodontol* **6:** 197–208.

Wada N, Maeda H, Tanabe K *et al* (2001). Periodontal ligament cells secrete the factor that inhibits osteoclastic differentiation and function: the factor is osteoprotegerin/osteoclastogenesis inhibitory factor. *J Periodontal Res* **36:** 56–63.

Wohlfahrt JC, Wu T, Hodges JS, Hinrichs JE, Michalowicz BS (2006). No association between selected candidate gene polymorphisms and severe chronic periodontitis. *J Periodontol* **77:** 426–436.

Wynne F, Drummond F, O'Sullivan K *et al* (2002). Investigation of the genetic influence of the OPG, VDR (Fok1), and COLIA1 Sp1 polymorphisms on BMD in the Irish population. *Calcif Tissue Int* **71:** 26–35.

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.

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.