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Single Nucleotide Polymorphisms in the Human Gene for Osteoprotegerin are Not Related to Bone Mineral Density or Fracture in Elderly Women

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Abstract. Osteoprotegerin (OPG), a secreted member of the tumor necrosis factor receptor family, is a potent inhibitor of osteoclast activation and differentiation. In animal models OPG prevents bone loss, and in humans bone resorption can be reduced by injections of OPG. OPG may also play a role in cardiovascular disease since mice lacking the OPG gene display arterial calcification. In a screening effort of the OPG gene, we recently discovered a single nucleotide polymorphism in the promoter region of OPG (T950C), and reported an association with vascular morphology and function in 59 healthy individuals. Due to the pronounced effect of OPG on bone turnover, the present study was conducted to investigate whether OPG polymorphisms are also associated with bone mineral density or with fracture. The relationship between single nucleotide polymorphisms in the promoter region of OPG (T950C) and the first intron (C1217T), and bone mineral density, measured by DXA in the hip or spine or ultrasound of the heel, was investigated in the Malmö OPRA-study of 1044 women, all 75 years old. The possible relation to fracture incidence was also analyzed. Among the 858 and 864 individuals respectively, genotyped, no significant associations between the investigated single nucleotide polymorphisms and bone mineral density measurements (T950C $P = 0.50$–0.64, C1217T $P = 0.51$–1.00), quantitative ultrasound measurements of the calcaneus, or fractures (T950C $P = 0.61$–0.66, C1217T $P = 0.14$–0.33) were found. Thus, our results show that polymorphisms in the OPG gene, one of which has previously been found to be associated with cardiovascular morphology and function, are not associated with bone mineral density in elderly Swedish women.

Key words: OPG — Polymorphism — BMD — Ultrasound — Fractures

Twin and family studies have demonstrated that approximately 80% of the variation in bone mineral density (BMD) is determined by genetic factors [1–3]. The genes contributing to BMD remain to be elucidated, but potential candidate genes are those with regulatory effect on bone mass such as collagen type I, estrogen, and vitamin D receptors and various growth factors and cytokines [4]. A novel candidate gene is osteoprotegerin (OPG), a member of the tumor necrosis factor receptor family acting as a paracrine factor in the bone microenvironment to decrease bone resorption [5–7]. *In vivo* over-expression of OPG in transgenic mice resulted in a nonlethal osteopetrosis and decreased osteoclast differentiation [5]. The OPG −/− mice exhibited a decreased total bone density and a high incidence of fractures [8, 9]. These findings suggest that levels of OPG can play a crucial role in bone remodelling. However, the OPG −/− mice also showed a presence of arterial calcification of the aorta and renal arteries [8] and a more recent study showed that OPG could inhibit warfarin- and vitamin D-induced vascular calcification in rats *in vivo* [10]. This indicates that OPG may also play an important role in vascular biology.

The human OPG gene was cloned and characterized by Morinaga et al. in 1998 [11]. It is a single copy gene on chromosome 8, with 5 exons that spans 29 kb of the human genome [11]. By sequence analysis we recently discovered two single nucleotide polymorphisms (SNP), one in the promoter region of the OPG gene and one in intron 1. The promoter T/C SNP is located at position 950 (T950C) and the T/C SNP in intron one is located at position 1217 (C1217T), 15 bp downstream of exon 1, according to the published sequence by Morinaga et al. We have previously studied the promoter polymorphism for association to vascular morphology and function due to the interesting finding in the OPG −/− mouse. An association between OPG genotype and increased intima media thickness in the common carotid artery, which is believed to be an index for early atherosclerosis and increased forearm blood flow, a measurement of vasodilatory capacity, was found in 59 healthy individuals [12–14], Also, we could confirm this finding in 100 hypertensive individuals where we found an association between increased intima-media thickness of the com-

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mon carotid artery and OPG CC genotype (Brändström et al. unpublished data).

Since OPG has dramatic effects on bone remodelling and since administration of OPG has been suggested as a potential pharmacological agent for osteoporosis, we wanted to investigate the potential association of these polymorphisms with bone mass and fracture in elderly women. Our primary focus was the T950C promoter polymorphism because of the functional implication. Secondly, we investigated the C1217T SNP located in the first intron which has at present no known functional effect and has not previously been studied in relation to osteoporosis.

Subjects and Methods

Subjects

The Malmö Osteoporosis Prospective Risk Assessment (OPRA) study cohort includes 1044 women, all 75 years old. In this population-based study, aimed at identifying risk factors for fracture, 1604 women were randomly selected from the city files and invited by letter. A total of 1044 (65%) responded, all of Caucasian background, 12 of whom had immigrated from other European countries. In all, 560 women did not participate, 13 of whom died shortly after the invitation, 139 could not come due to illness, 376 were not interested or could not attend due to reasons other than illness, and 32 were not reached despite repeated letters and phone calls.

Blood samples were obtained from 969 women. The OPG genotypes were determined in 858 and 864 women, respectively. Lack of genotype information was due to inadequate amounts of genomic DNA, or PCR failure. These 864 individuals are included in the present analysis, providing they have had valid bone mass measurements from at least one site.

DNA Analysis

Leukocyte DNA from each individual was extracted from 3 ml whole blood using a Wizard blood kit (Qiagen GmbH, Promega, Madison, USA). For the T950C SNP, a 330 bp fragment corresponding to the osteoprotegerin promoter region (gene-bank accession number AB008821) was amplified by polymerase chain reaction (PCR) using the following primer pair: 5′-CCCAGG GGACAGACACCAC-3′ (forward), and 5′-GCGCGCAGCACAGCAACTT-3′ (reverse). PCR reactions were run on a Gene Amp PCR system-9700 robot using Ampli-Taq Gold® kits and standard reagents (Perkin Elmer Co, Nonwalk CT, USA.). The amplification profile for the 330 bp segment of the OPG promoter consisted of denaturation at 96°C for 10 min, followed by 36 cycles with denaturation at 96°C for 30 sec, annealing at 63°C for 30 sec and elongation at 72°C for 1 min, and final extension at 72°C for 7 min. A second PCR for the C1217T SNP resulting in a 298 bp fragment was performed with primers 5′-GCAGGCGATACTTCCTGTT G-3′ (forward) and 5′-GTTTCCTGCTCCAGCCTAAC-3′ (reverse), using the same PCR conditions as above, but an annealing temperature of 57°C.

PCR-restriction Fragment Length Polymorphism Analysis

The PCR fragments containing the T950C and C1217T SNPs were digested with 10 U of Hinc-II or Hae III, respectively (Life Technologies, Stockholm, Sweden), at 37°C for 2 hours followed by staining with ethidium bromide and separation on a 1.5% agarose gel.

DXA Measurements

BMD measurements were made with a LUNAR DPX-L scan (Lunar corporation, Madison, WI, USA) of the hip (neck and trochanter) region and lumbar spine (vertebrae L2-L4) (g/cm^2). By double measurements in 14 healthy individuals, the precision of the DXA measurements in our laboratory has previously been determined to 0.5% (lumbar spine), 1.6% (femoral neck), and 2.2% (trochanter) [15]. The stability of the DPX-L equipment was checked every morning using a phantom. The long-term precision of the apparatus during 3–4 years follow-up was calculated by daily measurements of a special referee phantom to 0.48% during the two measurements. Of the total number of women included, DXA of the spine was available in 974 women and of the hip (neck and trochanter) in 951 women.

Ultrasound Measurement of the Calcaneus

Quantitative ultrasound of the calcaneus (QUSc) was performed with a Lunar Achilles® (Lunar Corporation, Madison, WI, USA) of the right calcaneus (if previous injury or fracture, the left calcaneus was used). The result was given as speed of sound and broadband ultrasound attenuation. The precision of the ultrasound equipment in our laboratory, as assessed by double measurements in 14 healthy persons after repositioning, has previously been determined to be 1.5% [16]. The long-term stability of the apparatus was controlled for by daily calibration with two phantoms provided by the manufacturer. Quantitative ultrasound of the calcaneus (QUSc) was performed in a total of 853 women.

Ultrasound Measurement of the Phalanges

QUS of the phalanges (QUSp) was performed on the non-dominant hand with a DBM Sonic 1200® (IGEA, Carpi, Mo, Italy) and measures amplitude-dependent speed of sound (m/s). This instrument has a probe that emits ultrasound, and is placed over the proximal phalanx just proximal to the distal metaphysis. Ultrasound gel is used to exclude air from the measurement site. The second to fifth fingers are measured once and the mean is used. The women (909) were measured (883 left side, 26 right side) of the 1044 originally invited. The inter- and intra-observer precision at our laboratory, determined in aged women, is 2.5% [17].

The main reasons for attending women not being examined for bone mass with one or any method was instrument failure, high body weight, disability not allowing supine position for the time required for measuring, or prior surgery interfering with the measurement.

Questionnaire

A comprehensive questionnaire was used for health and functional assessment. The questionnaire included questions on present physical activity (1–8; 1 = bed rest only, cannot walk, 8 = still working, no limitation of mobility), previous fractures and current drug intake.

Statistical Analysis

The values are presented as mean ± one standard deviation, unless otherwise stated. For differences of the continuous variables between genotype groups, ANOVA, General Linear Models ANOVA or *t*-test, as appropriate, compared the means. For categorical variables the Chi-square test was used.

Table 1. No difference in baseline characteristics, bone mass measurements, or possible confounders in any of the T950C genotypes (total n = 858 women)

Genotypes	n	TT (mean SD)	TC (mean SD)	CC (mean SE)	P^a
No. of individuals		227	421	210	
Age (yr)	858	75.2 (0.16)	75.2 (0.14)	75.2 (0.14)	0.15
Weight (kg)	858	68.8 (13.4)	68 (10.7)	68 (10.9)	0.67
Height (m)	846	1.6 (5.9)	1,61 (5.7)	1.6 (5.5)	0.55
BMI (kg/m^2)	846	26.7 (4.9)	26.2 (3.8)	26.5 (4.1)	0.43
Age at menopause	834	49.1 (4.7)	49 (5.0)	49.3 (5.1)	0.89
Physical activity (1–8)	853	5.7 (1.0)	5.7 (1.1)	5.7 (1.0)	0.94
OUS					
SOS (m/s)	718	1502 (26)	1503 (27)	1503 (26)	0.83
BUA (dB/MHz)	718	99 (10.2)	99.3 (9.8)	99.7 (9.0)	0.79
Stiffness	718	66.5 (12.9)	67.4 (11.7)	67.4 (11.7)	0.77
Phalanges (m/s)	753	1795 (97)	1797 (94)	1797 (96)	0.95
		n	n	n	P^b
Drug intake					
Estrogen		None	None	None	
Bisphosphonate		None	None	None	
Calcium[c]		5	11	7	0.76
Vitamin D[d]		7	7	3	0.37
Antihypertensives		87	170	75	0.52
Cortisone		None	None	None	
Smoking					
Current smokers		32	54	32	0.70

n = number of women, if less than 858 due to missing data
[a] *P*-value for ANOVA tests
[b] *P*-value for Chi^2 tests
[c] Calcium tablets containing 500 mg or more of Ca^{2+}
[d] Vitamin D tablet containing 400 units or more of vitamin D_3

For the C1217T SNP, the homozygotic TT genotype only included 2 individuals; these are excluded from the calculations and comparison includes the heterzygotic CT and homozygotic CC groups. The level of significance was accepted as *P*-values < 0.05. All statistical calculations were performed using STATISTICA (StatSoft^{TM}, Tulsa, OK, USA). Tests for Hardy-Weinberg equilibrium and linkage disequilibrium were performed using Arlequin ver 2.000 (http://lgb.unige.ch/arlequin/).

Results

The presence of a C allele at the T950C SNP gives rise to a restriction recognition site for Hinc-II, and the OPG genotype of each individual could therefore be determined by RFLP. The allele frequencies in the cohort were as follows: 227/858 (26.5%) were homozygous for the TT genotype, 421/858 (49%) were heterozygous and 210/858 (24.5%) were homozygous for the CC genotype. The OPG allele frequencies were in agreement with a Hardy-Weinberg equilibrium ($P = 0.59$) and with the genotype frequencies we previously determined in the cohort of cardiovascular morphology and function [18]. The clinical characteristics of the cohort are shown in Table 1. We found no significant differences between the genotype groups with respect to age, body mass index (BMI) or age at menopause in the cohort.

Similarly, the presence of a C allele gives rise to a restriction recognition site for Hae-III restriction enzyme at the C1217T SNP. The allele frequencies for this SNP in the cohort were as follows; 2/864 (0.2%) were homozygous for TT, 93/864 (10.7%) were heterozygous, and 769/864 (89.0%) were homozygous for CC. The allele frequencies were in agreement with Hardy-Weinberg equilibrium ($P = 1.00$) and the genotypes were in strong linkage disequilibrium ($P = 7.825 \times 10^{-10}$). Clinical characteristics were similar between the genotype groups (data not shown).

Genotype and Association to Bone Mass

There was no significant association between OPG T95C genotype and BMD at femoral neck ($P = 0.60$), trochanter ($P = 0.50$) or lumbar spine ($P = 0.64$) in these elderly women (Fig. 1). Nor was there any difference in the QUSc measurements between genotypes (Table 1). According to the DXA results (femoral neck, trochanter or spine) the women were divided into quartiles (quartile size n = 197-200). The highest quartile of DXA femoral neck represents women with a T-score > -1.27 and the lowest quartile women with a T-score < -2.67 and thus, clearly osteoporotic. The corresponding figures for the trochanter were > 0.06 and < -1.71, and for spine > -0.87 and < -2.80. The

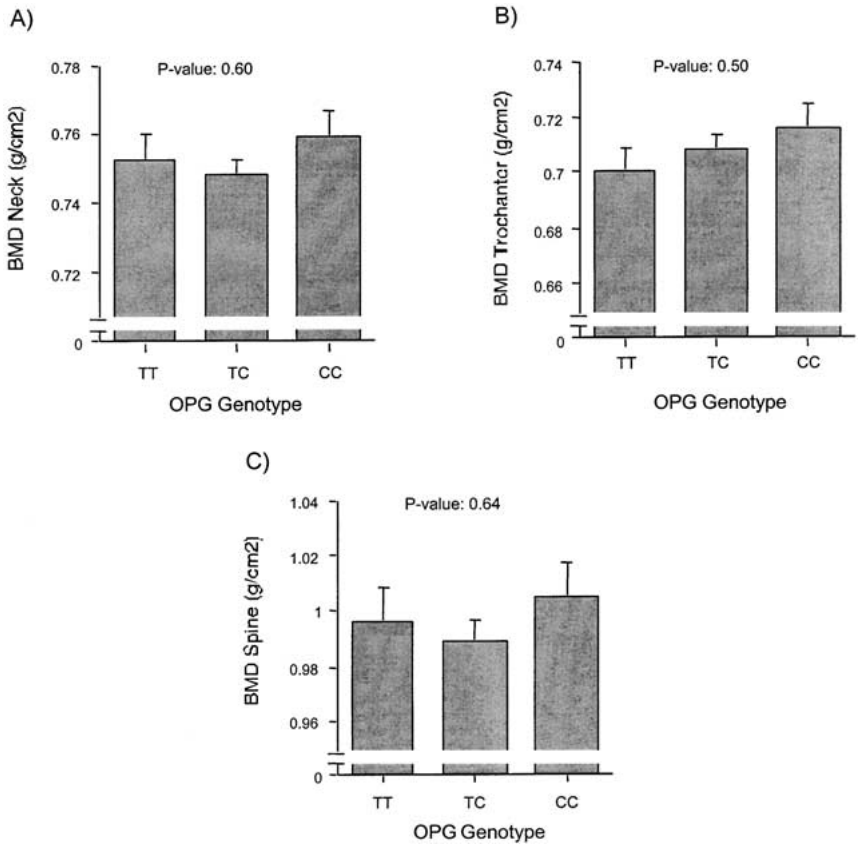

Fig. 1. Distribution of BMD and OPG T950C genotype in the female cohort. No statistically significant differences in bone mineral density among the different OPG-genotypes was seen. Data are presented as means ± SEM for the three genotype groups. For hip (femoral neck and trochanter) measurements, group TT consists of 204 (26%) of the individuals, TC 388 (49%) of the individuals and CC 197 (25%) of the individuals. For lumbar spine the numbers are 208 (26%), 396 (49%) and 199 (25%), respectively. (**A**) BMD measurements for femoral neck, (**B**) BMD measurements for trochanter, (**C**) BMD measurements for lumbar spine, L2-L4.

genotype distribution was studied within and between quartile groups and there was no association between genotype and BMD.

For the OPG C1217T genotype, no association was evident for bone mass, neither measured by DXA (femoral neck $P = 0.51$, trochanter $P = 0.63$, spine $P = 1.00$) nor by ultrasound (QUSc $P = 0.14–0.33$, QUSp $P = 0.22$).

Genotype and Association to Fracture

No association was found between OPG T950C genotypes and women with at least one fracture during lifetime (n = 361; 42%) as compared to women without earlier fractures ($P = 0.61$). In a subgroup analysis, fractures having occurred after the age of 50 were studied as indicators of fractures of a potentially osteoporotic origin. No association was found between women having sustained at least one fracture after the age of 50 (n = 295; 34%) compared to women with no fracture after the age of 50 ($P = 0.66$) and OPG genotype. Identification of women with fractures specifically related to osteoporosis, wrist, vertebral and hip fractures gave similar results ($P = 0.11–0.80$) (Table 2). The OPG C1217T genotype was not associated with fracture in this cohort of elderly women ($P = 0.12–0.96$). Women with any previous fracture and those with a previous fracture of the hip and wrist had significantly lower BMD and QUSc (1–20% lower) than women with no previous fracture ($P = 0.01$ or less), while the lower BMD value (3–6% lower) was not significant in women with vertebral fractures ($P = 0.12–0.50$).

Possible confounding factors identified through the questionnaire that could affect bone mass (BMI) (smoking, drug intake and physical activity) was also evaluated, but no differences between the T950C genotype groups were found (Table 1).

Discussion

The pathogenesis of osteoporosis is complex and includes both genetic factors and environmental influences. As a consequence, bone cell function is affected by altered production of local and systemic hormones with the capacity to modulate bone turnover. Peak bone mass and the rate of bone loss after menopause are two key factors likely to be influenced by genetic determinants. Considering the *in vitro* and *in vivo* studies that have shown potent effects of OPG on bone cells [19–21], this protein has the capacity to serve as an important regulator of bone resorption and thereby genetic variations in the OPG gene may influence BMD. OPG deficiency leads to increased osteoclastogenesis from diminished competitive inhibition of RANKL, a pow-

Table 2. The T950C genotype distribution in relation to fracture sustained before baseline examination

	Genotype			
	TT	TC	CC	Total N
At least one fracture during life time	93	182	86	361
At least one fracture after age 50	79	145	71	295
Hip fracture	14	19	4	37
Wrist fracture	34	83	37	154
Vertebral fracture	5	13	1	19
No fracture during life time	134	239	124	497

erful stimulator of osteoclast differentiation and activity. Effects of OPG are not limited to bone cells; we recently detected a polymorphism in the promoter region of OPG (T950C) that strongly correlates with measurements of vascular function and morphology. A logical next step was to investigate the possibility of an association between this polymorphism and bone density.

This is of interest for several reasons: first, the polymorphism reported by us is situated in the promoter region of the gene, which could theoretically affect the levels of OPG expression through transcriptional regulation. Furthermore, osteoporosis has been associated with a higher incidence of arterial calcification [22, 23]. Also, epidemiological studies have suggested a relationship between osteoporosis and atherosclerosis [22, 24] even though contradictory results have been reported on this aspect [25, 26]. The T950C promoter polymorphism has been studied in relation to cardiovascular effect. Our group reported an association between the CC genotype of the OPG SNP and high intima media thickness, a sign of early atherosclerosis [18] and a decreased forearm blood flow, an index of vasodilatory capacity [12–14]. Also the serum levels of OPG seem to be associated with cardiovascular disease. In a cohort of 490 women over the age of 65, serum OPG was associated with cardiovascular mortality [27]. Recently, OPG levels have also been found to be associated with severity of cardiovascular disease [28].

These findings indicate that OPG is involved in, or serves as a marker for vascular calcification, but whether it also serves as a marker for bone density is less clear. In the above-mentioned study of women, serum OPG was not associated with bone mineral density or fracture [27], whereas an inverse relationship among bone density, bone resorption and serum OPG was suggested to be present in men [29, 30]. Several other polymorphisms have been identified in the OPG gene, although the limited number of studies have shown inconsistent association to bone [31]. In addition to the OPG T950C polymorphism, we also identified a polymorphism in the first intron, C1217T, without previously described functional or morphological associations to bone or other tissue. This therefore was the secondary SNP analyzed in this study. This polymorphism has been shown to be in complete linkage with two promoter polymorphisms, G209A and T245G, in a study of 595 Danish women [31].

In this study, representing a large cohort of elderly women, we could not detect any association between the SNP in the promoter region of OPG and BMD at relevant sites. Nor did we find any correlation to ultrasound measurements of the calcaneus or to reported fracture. Furthermore, we found no association between the SNP in the first intron and bone mass or fracture. QUS of the phalanges has in one prospective study of younger women (mean age of 52) been able to predict fractures [32] but contradictory results exists [17] and bone mass at this site was not associated to genotype differences.

The Malmö OPRA study used in this investigation is a well-characterized cohort of women exactly 75 years old. The lack of association between the OPG genotype and bone mass measured at multiple sites and by several methods may be due to other factors affecting bone in the elderly women, and it is possible that an association would be more readily seen in younger women, either at peak bone mass or in peri- or early postmenopausal women. However, we believe that our negative findings in these elderly women are reliable, since no correlation was evident for bone mass measured at any site. Furthermore, in the subgroup analysis of quartiles, where the lowest quartile represented clearly osteoporotic subjects, the polymorphism was not distributed differently. Our findings are in line with a study of younger postmenopausal women in which the T950C was unrelated to femoral neck or spinal BMD in a healthy control population ($n = 327$), but associated with BMD of the lumbar spine in osteoporotic women ($n = 258$) [31]. Several polymorphisms of the OPG gene were investigated in this study, while no consistent associations to BMD were found. In an Irish study of the same polymorphism in pre- and postmenopausal women and limited to femoral neck and spine BMD, no association was found, although the genotype distribution was similar [33]. On the other hand, an association was found in a group of Japanese women, however, they were only evaluated by distal forearm BMD [34]. Among the few reports, all including smaller populations of lower mean age, association to bone mass has been reported for the polymorphisms G209A, T245G and G1181C; again the findings have been inconsistent and depending on measured site and the population [31, 34, 35]. Although we also investigated C1217C, which is in linkage with the G209A and T245G polymorphisms, we did not find any associations to bone. The inconsistency clearly indicates that associations related to variation in the OPG gene and the phenotypic expressions of bone mass and fracture exhibit differences related chiefly to age, ethnic and geographic factors, which

allow for variations to have a different impact in different populations.

Fracture is only partially dependent to bone density, and additional factors are of importance for bone strength, but also the impact of the trauma plays a key role. In these women, there was no association between OPG genotype and fracture sustained at any time during life or when specifically identifying fractures that are of potentially osteoporotic origin. The information on fractures is self-reported and thereby a possible error in under- or over-reporting could mask an association; nevertheless, a pronounced association seems unlikely.

Potential confounders, masking possible associations to bone mass in the elderly, are degenerative changes of the lower spine or calcifications of major vessels. This may also be the case in the present study. However, we have extensively evaluated bone mass by using several methods and measuring sites, where a possibly qualitative measure, particularly in QUS, has not previously been evaluated in relation to OPG genotype.

In conclusion, the SNP in the promoter region of OPG previously reported to be associated with cardiovascular morphology and function does not influence bone mass or fractures in elderly Swedish women. Nor was the previously non-studied polymorphism in the first intron of OPG found to be associated with bone mass or fracture in this large cohort. More studies on the role of OPG in cardiovascular and bone disease are warranted, preferably in cohorts of different age and different outcomes, such as hypertension and myocardial infarction, as well as BMD and fractures.

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