Is A163G Polymorphism in the Osteoprotegerin Gene Associated with Heel Velocity of Sound in Postmenopausal Women?

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SHORT TITLE: OSTEOPROTEGERIN POLYMORPHISMS AND QUANTITATIVE BONE ULTRASOUND

Summary

Osteoprotegerin (OPG) plays an important inhibitory role in osteoclastogenesis. Polymorphisms in the *OPG* gene recently have been associated with various bone phenotypes including fractures. The aim of the present study was to investigate the association between three informative *OPG* polymorphisms and quantitative ultrasound variables of the heel. In a cohort of 165 perimenopausal women polymorphisms in the *OPG* promoter (A163G, T245G) and in exon 1 (G1181C) were assessed by PCR-RFLP analysis. The distribution of the investigated genotypes was similar to other Caucasian women (A163G-AA 68%, AG 30%, GG 2%, T245G-TT 84,4%, TG 15%, GG 0,6%, G1181C- GG 22%, CG 55%, CC 23%). After adjustment for body mass index and years since menopause, in a subgroup of 87 postmenopausal subjects, calcaneal velocity of sound (VOS, m/s) was significantly associated with A163G polymorphism (p=0.0102, ANCOVA). Women with the presence of G allele (AG+GG genotypes) had significantly lower VOS than women with AA genotype. Neither T245G nor G1181C were associated with calcaneal ultrasound indices. In conclusion, A163G polymorphism was significantly associated with VOS at the heel in a limited cohort of postmenopausal women. The present study replicated in part the previous findings about *OPG* gene variations and peripheral bone mass in Caucasian women.

Key words

osteoprotegerin gene - polymorphisms - VOS - BUA - menopause

Introduction

Calcaneal quantitative ultrasound (QUS) provides two measures: velocity of sound (VOS, m/s) and broadband ultrasound attenuation (BUA, dB/MHz). In vitro studies of cancellaneous bone have shown that BUA is associated with trabecular bone structure whereas VOS depends on elasticity and density of the bone (Bouxsein *et al.* 1997). Both ultrasound parametres are correlated with bone mineral density (BMD, g/cm^2). QUS parametres along with BMD predict osteoporotic fracture risk (Hartl *et al.* 2002). However, this association remains significant even after adjustment for BMD at particular sites of the skeleton. This fact indicates that QUS measurements may capture other aspects of bone structure and strength than bone densitometry (DXA) does (Lee *et al.* 2004).

Moreover, recent twin and family studies have indicated that a large proportion of QUS variability is determined by genetic factors. Heritable estimates for calcaneal BUA ranged from 0.5-0.7 and from 0.5 to 0.6 for VOS (Arden *et al.* 1996, Knapp *et al.* 2003, Lee *et al.* 2004).

Accelerated bone resorption contributes in large to postmenopausal bone loss. Candidate genes for local or systemic calciotropic factors involved in bone remodeling may play a role in the genetic variability of the bone phenotypes (Liu *et al.* 2006). Osteoprotegerin (OPG), a member of the tumor necrosis factor (TNF) and TNF receptor superfamily, plays an important inhibitory role in osteoclastogenesis. The activation and differentiation of osteoclasts through receptor activator of nuclear factor-κB ligand (RANKL) is neutralized by the decoy receptor OPG (Khosla 2001). Injected OPG can thus prevent bone resorption in humans (Khosla 2001). Various upstream regulators of the bone metabolism (e.g. estrogens, leptin) induce an imbalance between OPG and RANKL activation which results in either bone loss or accrual (Khosla 2001).

Sequence variations in the genes involved in the RANKL/RANK/OPG bone remodeling pathway might be, therefore, associated with different bone phenotypes (Gallo *et al.* 2007).

The *OPG* promotor region represents a target sequence for various calciotropic factors regulating *OPG* gene expression. Polymorphisms in this and nearby regions may contribute to genetic regulation of bone mass. Various single nucleotide polymorphisms (SNP) in the *OPG* gene have been inconsistently associated with BMD or fractures (Arko *et al.* 2002, Langdahl *et al.* 2002, Jorgensen *et al.* 2004). A recent study of Caucasian postmenopausal women has demonstrated that A163G polymorphism, localized in the *OPG* promotor, has been related to peripheral indices of bone mass (Jorgensen *et al.* 2004).

The aim of the present study was to investigate the association between three informative polymorphisms in the regulatory region of the *OPG* gene and quantitative ultrasound variables of the heel in a cohort of postmenopausal women.

Methods and materials

Population

The genotyping was carried out in a cohort of 165 peri- and postmenopausal unrelated women of Caucasian origin. A subset of 113 ambulatory postmenopausal women (62.4 ± 8.97 years of age and 13.3 ± 8.35 years since menopause (YSM)) underwent QUS measurements (Table 1).

The women were recruited through the screening of diseases associated with menopause including osteoporosis. None of the investigated women had history of early or late menarche or menopause. All subjects had normal calcium and protein intake; none was markedly underweight or obese, with the mean body mass index (BMI) 25.7 ± 3.51 kg/m^2 (Table 1). The study group

did not include any alcoholics, heavy smokers, women with active endocrinopathy or severe internal disease or those treated with calciotropic drugs including estrogen and vitamin D.

Informed consent was obtained from all of the women, and all procedures were approved by the Ethical Committee of the Institute of Endocrinology, Prague.

Calcaneal quantitative ultrasound measurements

Calcaneal QUS was measured at the right heel using a dry contact ultrasound scanner (CubaClinical, UK). The coefficient of variation for BUA was 3.04 %, for VOS 0.24% .The quantitative ultrasound index, called „stiffness index“, was calculated as a linear combination of BUA and VOS (Genant *et al.* 1996).

Genotyping

Genomic DNA was isolated from peripheral leukocytes by a standard salt extraction procedure (Miller *et al.* 1988). All SNPs were assessed by a restriction analysis of the PCR products as described elsewhere (Langdahl *et al.* 2002).

Stastistics

In both A163G and T246G polymorphisms, due to the low frequency of GG genotypes, the AG with GG and TG with GG genotypes were pooled together, respectively. Differences in QUS measures within the *OPG* genotypes were evaluated by ANCOVA after adjustment for BMI and YSM. Statistics were done using the Statgraphics plus v.5.1 software from Manugistics (Rockville, MA, USA). Data are expressed as median and lower-upper quartile in Table 3 or as

mean ± SD in Table 1. Chi-square (χ^2) formula was used to test for Hardy-Weinberg equilibrium and to detect differences from the expected frequencies.

Results

The distribution of the investigated genotypes did not deviate from Hardy-Weinberg equilibrium and was similar to other Caucasian women (Table 2) (Langdahl *et al.* 2002, Jorgensen *et al.* 2004, Ueland *et al.* 2007). In a subgroup of 87 women who underwent QUS measurements, A163G polymorphism was significantly associated with calcaneal VOS (p=0.0102, ANCOVA)(Table 3). Women with the presence of G allele (AG+GG genotypes) had significantly lower VOS than women with AA genotype. The statistic analysis was carried out after adjustment for BMI and YSM. Similar results between A163G and BUA were observed, however, this trend did not reach significance (Table 3).

Neither T245G nor G1181C were associated with any calcaneal ultrasound indices (data not shown).

Discussion

In the present study we investigated three informative polymorphisms from the regulatory region of the *OPG* gene including promotor A163G and T245G variations with a nonsynonymous polymorphism in exon 1 (G1181C). A significant association between A163G polymorphism and calcaneal VOS was found in the present limited cohort of postmenopausal women.

Twin and family studies have shown that QUS measures are highly heritable (Arden *et al.* 1996, Lee *et al.* 2004, Liu *et al.* 2006). Menopause seems to play a lesser role in overall QUS

variability in comparison with BMD (Hunter *et al.* 2001). Additionally, underlying genetic factors contributing to QUS variations might be in part unique, unshared with those involved in BMD regulation (Knapp *et al.* 2003, Lee *et al.* 2006).

Several recent publications have addressed the hypothesis that polymorphisms in the regulatory region at the 5´ end of the *OPG* gene may contribute to the genetic regulation of various bone phenotypes. In a study of Lagdahl et al. (2002) the *OPG* gene has been sequenced and consequently 12 polymorphisms have been described. The majority of them have been, however, in a complete linkage. For further analysis, five informative polymorphisms have been selected. Genotypes with the rare G allele (A163G and T245G) have significantly prevailed in patients with vertebral fractures in comparison with controls. In the same cohort, these genotypes have not been related to BMD or biochemical markers of bone turnover (Langdahl *et al.* 2002). These findings implicate that *OPG* polymorphisms might be associated with bone quality parametres others that bone mineral density or biochemical turnover. A significant association between A163G polymorphism, hip and wrist fractures has been confirmed in another study in Caucasian postmenopausal women (Jorgensen *et al.* 2004). Moreover, A163G genotypes have been associated with low peripheral bone mass assessed either by DXA at the distal radius or by QUS at the heel. In genotypes with unfavourable G allele lower BMD, calcaneal BUA and VOS have been found. The effect of A163G polymorphism on bone mass was not mediated through serum OPG levels as might be expected (Jorgensen *et al.* 2004). A twin study has demonstrated that, unlikely BMD or QUS, circulating OPG levels were almost exclusively determined by environment (Abrahamsen *et al.* 2005). These findings, however, do not exclude that local, paracrine OPG concentrations may vary within the bone microenvironment between the *OPG* genotypes. Functional in vitro analysis might therefore clarify why the presence of G allele (A163G), in particular, has been associated with deteriorated bone phenotypes.

On the other hand, negative associations on *OPG* polymorphisms have also been presented. In a large group of elderly Australian women of Caucasian origin none of the *OPG* polymorphisms have been associated with any of the DXA or QUS measurements (Ueland *et al.* 2007). The study, which had negative results, was carried out on a well-described large cohort of Australian elderly women, thus providing sufficient power to detect minor genetic contribution to overall trait variability. Different genetic background of investigated cohorts might explain divergent results.

In the present study a significant association between A163G polymorphism and VOS was demonstrated. Subjects with the presence of G allele (AG+GG genotypes) had significantly lower VOS than women with AA genotype. Similar results were found for BUA, however this trend did not reach statistical significance. These discordant findings probably do not have any physiological relevance but may very likely reflect false negative results due to the limited size of the study cohort (Brown 2005). Further studies are therefore necessary in a larger general population to confirm the relationship between A163G polymorphism and bone ultrasound indices.

In conclusion, A163G polymorphism in the *OPG* gene was significantly associated with VOS at the heel in a small, well-characterized cohort of postmenopausal women. The present study replicated in part the previous findings about *OPG* gene variations and peripheral bone mass in Caucasian women.

There is no conflict of interest.

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Table 1. Baseline demographic and quantitative ultrasound characteristics of the study population

Demographic data	n	
Age (years)	113	62.4 ±8.97
YSM (years)	113	13.3 ±8.35
BMI	113	25.7 ±3.51
Calcaneal quantitative ultrasound		
BUA (Db/MHz)	113	66.9 ±18.5
VOS (m/s)	87	1603 ±50.8
Stifness index	87	73.6 ±22.8

Results are given as mean ± SD.

Table 2. A163G, T245G and G1181C polymorphisms in the OPG gene: genotype distribution, allele frequency and parametres of Hardy-Weinberg equilibrium in a cohort of 165 perimenopausal women

SNP		A163G			T245G			G1181C	
dbSNP reference number		rs3102735			rs3134069			rs2073618	
Genotype distribution	AA	112	68%	TT	139	84.4%	GG	37	22%
	AG	50	30%	TG	25	15%	CG	90	55%
	GG	3	2%	GG	1	0.6%	CC	38	23%
Allele frequency and Hardy-Weinberg equilibrium	A	0.83	$\chi^2 = 0.963$	T	0.92	$\chi^2 = 0.014$	G	0.497	$\chi^2 = 1.356$
	G	0.17	$\pi \geq 0.50$	G	0.082	$\pi \geq 0.90$	C	0.503	$\pi \geq 0.50$

Table 3. Calcaneal quantitative ultrasound parameters in relation to the A163G genotypes

	A163G				
	n	**AA**	n	**AG + GG**	***p*** (ANCOVA)
BUA (Db/MHz)	77	66 (54-78)	36	58 (52.25-67.5)	NS
VOS (m/s)	63	1603 (1575-1636)	24	1569 (1540-1615)	0.0102
Stifness index	63	72.51 (61.07-84.04)	24	59.45 (49.04-76.58)	NS

Results are expressed as median (lower-upper quartile).
p values are by ANCOVA.