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ORIGINAL ARTICLE

Polymorphisms and haplotypes across the osteoprotegerin gene associated with bone mineral density and osteoporotic fractures

S. Jurado · X. Nogués · L. Agueda · N. Garcia-Giralt · R. Urreizti · G. Yoskovitz · L. Pérez-Edo · G. Saló · R. Carreras · L. Mellibovsky · S. Balcells · D. Grinberg · A. Díez-Pérez

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Abstract
Summary Osteoprotegerin plays a key role in bone remodelling. We studied the association between 24 polymorphisms and haplotypes on the *OPG* gene and bone mineral density and fractures. After multiple-testing correction, one SNP and two block-haplotypes were significantly associated with FN BMD. Two other block-haplotypes were associated with fracture.

S. Jurado · X. Nogués · N. Garcia-Giralt · G. Yoskovitz · L. Mellibovsky · A. Díez-Pérez
Internal Medicine, URFOA, IMIM, RETICEF,
Hospital del Mar, Autonomous University of Barcelona,
Barcelona, Spain

X. Nogués
e-mail: XNogues@imas.imim.es

N. Garcia-Giralt
e-mail: ngarcia@imim.es

G. Yoskovitz
e-mail: guy.yoskovitz@gmail.com

L. Mellibovsky
e-mail: LMellibovsky@imas.imim.es

A. Díez-Pérez
e-mail: ADiez@imas.imim.es

L. Agueda · R. Urreizti · S. Balcells · D. Grinberg
Departament de Genètica, Facultat de Biologia,
Universitat de Barcelona,
Barcelona, Spain

L. Agueda
e-mail: lagueda@ub.edu

R. Urreizti
e-mail: roseruf@yahoo.es

S. Balcells
e-mail: sbalcells@ub.edu

D. Grinberg
e-mail: dgrinberg@ub.edu

L. Agueda · R. Urreizti · S. Balcells · D. Grinberg
Institut de Biomedicina de la Universitat de Barcelona (IBUB),
Barcelona, Spain

L. Agueda · R. Urreizti · S. Balcells · D. Grinberg
CIBER de Enfermedades Raras (CIBERER),
Barcelona, Spain

L. Pérez-Edo
Department of Rheumatology, URFOA, IMIM, RETICEF,
Hospital del Mar, Autonomous University of Barcelona,
Barcelona, Spain
e-mail: lperez@imas.imim.es

G. Saló
Department of Traumatology and Orthopaedic Surgery, URFOA,
IMIM, RETICEF, Hospital del Mar,
Autonomous University of Barcelona,
Barcelona, Spain
e-mail: Gsalo@imas.imim.es

R. Carreras
Department of Gynecology and Obstetrics, URFOA, IMIM,
RETICEF, Hospital del Mar,
Autonomous University of Barcelona,
Barcelona, Spain
e-mail: Rcarreras@imas.imim.es

S. Jurado (✉)
Institut Municipal d'Investigació Mèdica (IMIM),
Parc de Rercerca Biomèdica (PRBB),
Dr Aiguader 88,
08003 Barcelona, Spain
e-mail: susanaweiter@hotmail.com

Introduction and Hypothesis Osteoprotegerin (OPG) plays a key role in bone remodelling. Here we studied the association between polymorphisms and haplotypes on the *OPG* gene and bone mineral density (BMD) and fractures.
Methods Twenty-four single nucleotide polymorphisms (SNPs) were selected to cover six haplotypic blocks and were genotyped in 964 postmenopausal Spanish women. Haplotypes were established with HaploStats. Association was analysed by GLM (for BMD) and logistic regression (for fractures) both at single SNP and haplotype levels.
Results Upon adjustment for multiple testing ($p<0.0073$), one of the SNPs (SNP #17, rs1032129) remained significantly associated with FN BMD ($p=0.001$). Four block-haplotypes stood multiple-testing correction. Two remained associated with FN BMD and two with fracture. The association of block-4 haplotype "AC" (of SNPs #18 and #17) with FN BMD ($p=0.0002$) was stronger than that of SNP#17 alone and was the best result overall. A global assessment of the results indicated that all the alleles and haplotypes with a protective effect, at $p<0.05$, belonged to a frequent long-range haplotype.
Conclusions In conclusion, these results provide a detailed picture of the involvement of common variants and haplotypes of the *OPG* gene in bone phenotypes.

Keywords Association · BMD · Fracture · Haplotypic blocks · OPG · SNPs

Introduction

Osteoporosis is a complex disease with a strong genetic component and multiple association studies have pointed to candidate genes that might be involved in their pathogenesis [1, 2]. Osteoporosis is characterised by low bone mass, micro-architectural deterioration of bone tissue and bone fragility. These factors lead to an increased incidence of fractures. Bone remodelling is a complex cellular process that involves the resorption of bone by osteoclasts and the formation of bone by osteoblasts. An imbalance in this equilibrium results in metabolic bone disease such as osteoporosis.

Osteoprotegerin (OPG) is a member of the tumour necrosis factor receptor superfamily, which plays a key role in bone remodelling. Secreted by osteoblasts, OPG is a glycoprotein that acts as a soluble decoy receptor for the receptor activator of nuclear factor kappa B ligand (RANKL), located on the osteoblast membrane. OPG binding to RANKL blocks the interaction of the latter with the receptor activator of nuclear factor kappa B (RANK), on the osteoclast surface. The OPG-RANKL interaction inhibits osteoclast recruitment and activation, and induces osteoclast apoptosis [3, 4], thus playing an anti-resorptive role in bone. Overexpression of *OPG* in transgenic mice results in osteopetrosis, while *OPG* knock-out mice develops severe osteoporosis and arterial calcification [5, 6].

Analyses of the association between *OPG* single nucleotide polymorphisms (SNPs) and BMD, fracture or other phenotypes have given various results [7–22]. Most of these investigations obtained some positive results with SNPs in the promoter region. Interestingly, two of the studies, focusing on elderly women from Sweden [16] and Australia [20] failed to find association. Recently, the first two genome-wide association studies for bone mineral density (BMD) and fracture have been published [23, 24], in which five and two genome-wide significant loci were identified, respectively. In both studies, the *OPG* region was present. While these results clearly suggest a central role of *OPG* in the genetic determination of bone phenotypes, a detailed analysis of the polymorphisms and haplotypes within the locus is still not available.

The HapMap Project seeks to map and study the patterns of common genetic diversity in the human genome with the purpose to accelerate the search for the genetic causes of human disease. The genome can be structured into haplotypic blocks on the basis of the patterns of linkage disequilibrium (LD) and with only a few marker SNPs (tagSNPs) it is possible to capture most of the variation within a genomic region [25, 26].

OPG maps to chromosome 8, spans 28 kb and is divided into five exons [27]. In our study we structured the *OPG* gene and 9 kb of its promoter into haplotypic blocks to genotype a minimal number of SNPs and test their association with lumbar spine (LS) BMD, femoral neck (FN) BMD and osteoporotic fragility fractures in 964 postmenopausal Spanish women.

Materials and methods

Study sample

Participants were recruited from the Menopausal Unit of the Hospital del Mar, Barcelona. All the patients were consecutive, unselected, postmenopausal women attending the outpatient clinic for a baseline visit because of menopause. Patients were prospectively recruited regardless of their bone density values. Subjects with a history of bone disease, metabolic or endocrine diseases, hormone-replacement therapy, anti-resorptive or anabolic agents oral corticosteroids, antiepileptic drugs, lithium, heparin or warfarin treatments were excluded. Quantitative information on calcium intake, exercise, alcohol intake or smoking was not available. In the final cohort of 964 patients (all of Spanish ancestry), age, weight, height, age at menarche, age at menopause, years since menopause and months of breast-

feeding were recorded. Blood samples and written informed consent were obtained in accordance with the regulations of the Hospital del Mar Human Investigation Review Committee for Genetic Procedures. This group of patients is referred to as the BARCOS cohort [28, 29]. According to WHO criteria, 31.1% of the individuals had osteoporosis for LS and 36.55% had osteoporosis for FN BMD using NHANES reference values. The main characteristics of the participants are shown in Table 1.

BMD analysis and fractures

BMD (g/cm^2) was measured at the LS (L2–L4) and at the non-dominant FN. A dual-energy X-ray densitometer (QDR 4500 SL, Hologic, Whaltham, MA, USA) was used for measurements. In our centre, the technique has an in vivo coefficient of variation (CV) of 1.0% for LS and 1.65% for FN measurements. Non-vertebral and clinical vertebral fractures were recorded. Non-vertebral fractures were validated from medical records and spine X-ray was performed at baseline when there was a history of height loss or back pain. Fractures were defined as osteoporotic if they occurred after the age of 45, and were due to low-impact trauma (i.e. fall from standing height). Fractures of the face, fingers, toes and skull were excluded. Vertebral fractures were defined according to the semiquantitative criteria of Genant et al. [30].

DNA extraction

The buffy coats of 3 ml of blood, collected in EDTA tubes, were stored at −20°C. Genomic DNA was obtained from leucocytes by a salting-out procedure [31]. DNA concentration was measured by spectrophotometry (NanoDrop ND-1000 Spectrophotometer; NanoDrop Technologies).

Table 1 Characteristics of the BARCOS cohort

Variable	Mean±SD	*n*
Age (years)	55.47±8.73	958
Weight (kg)	64.53±10.00	964
Height (cm)	156.31±6.25	963
Years since menopause	7.75±8.61	950
Breast-feeding (months)	8.21±13.25	909
Menopause age	47.67±4.55	956
Menarche age	12.91±1.59	947
LS BMD (g/cm^2)	0.852±0.152	958
FN BMD (g/cm2)	0.672±0.115	525
Fracture	148 (18.7%)	
Vertebral	69 (46.62%)	
Hip	9 (6%)	
Wrist	32 (21.62%)	
Other non-vertebral	38 (25.67%)	
No fracture	642 (81.3%)	

LD Plot and selection of SNPs

Haplotypic blocks were established with CEU-HapMap data (Phase 2, published in October 2005). Blocks were defined by the confidence intervals method (Gabriel et al. [32]). In the Gabriel method, the haplotype block is defined as a region where less than 5% of comparisons, among informative SNPs, show evidence of historical recombination. SNPs were selected to tag major haplotypes within each block, in spite of their pair-wise LD. With this method, HapMap structures the osteoprotegerin gene and 9 kb of its promoter into six haplotypic blocks and 21 tagSNPs with minor allele frequencies (MAF) of at least 0.05. The genotyping of three tagSNPs was not feasible due to interferences with SNPlex probes and substitutes for them were searched. The final number of tagSNPs was 20 (Fig. 1a, b). Two non-synonymous SNPs were added to the collection (rs2073618, Asn3Lys, #7 and rs11573906, Val104Met, #23) and two other SNPs (rs2073617, T>C, #6 [7–11, 14, 19, 22], and rs3134069, A>C, #5 [7, 8, 11]), previously analysed by other authors, were also included. Table 2 shows the 24 SNPs selected.

Genotyping and SNP frequencies

Genotyping was performed by the SNPlex system (Applied Biosystems®). This system is a cost-effective large-scale genotyping technique that uses multiplexing (multiple reactions in a single tube or well) to rapidly identify large numbers of target genetic sequences. It allows for the simultaneous genotyping of 48 SNPs in a single biological sample. The analysis was carried out at the Centre de Regulació Genòmica (www.cegen.org). A previous accurate quantification of the DNA samples was performed by Picogreen (Invitrogen).

SNPlex performance at the CEGEN platform was evaluated by testing a total of 92 genomic DNAs and 521 SNPs and a reproducibility of 99.7% was achieved [33]. An average call rate of 95.77% was obtained for the 24 SNPs over 964 samples (min, 92.42%; max, 97.20%). To check for the quality of the genotyping, one SNP was typed by RFLP in 5% of the population. The results showed a 100% concordance between the two techniques

MAFs in the BARCOS cohort and in the HapMap Caucasian reference panel were compared and found to be similar (Table 2). The non-synonymous SNP rs11573906, with a HapMap MAF of 0.011, was non-polymorphic in our population. Three additional SNPs (rs12056490, #2; rs3134069, #5 and rs11573855, #10) had a MAF below

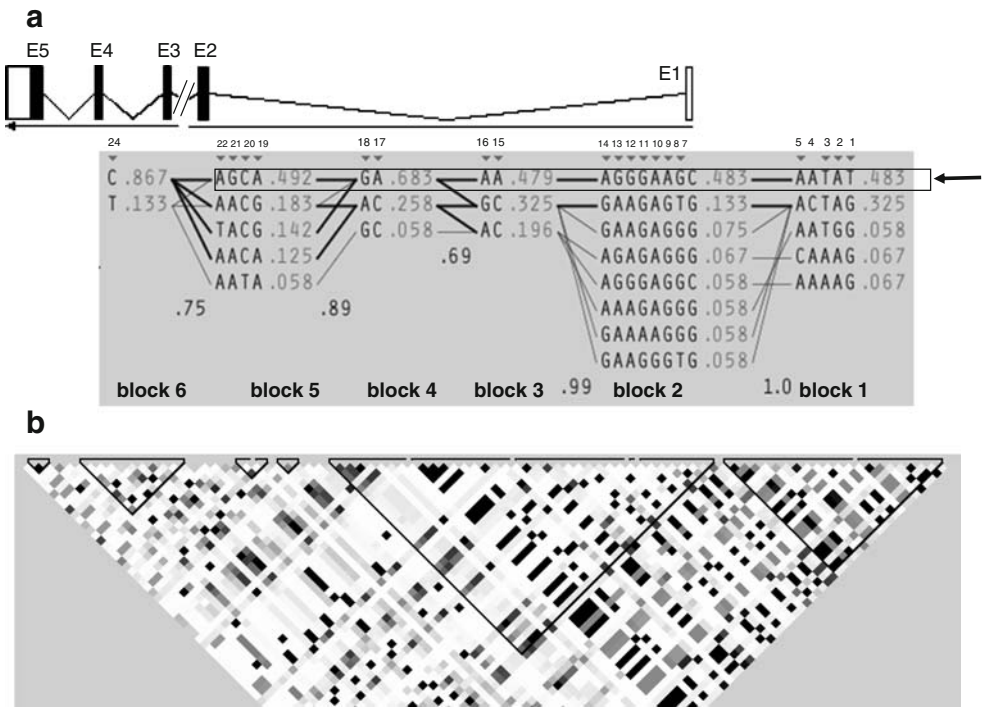

Fig. 1 *OPG* genomic and haplotypic structure. **a** Genomic structure (*top*) and haplotype blocks of CEU-HapMap data, following Gabriel et al. (*bottom*). The gene, depicted right to left to indicate that it is encoded on the minus strand, contains five exons. Haplotypic blocks were constructed with 22 of the 24 genotyped SNPs, 20 of which were tags (*triangle*). SNPs #6 and 23# are interblock SNPs and are not included in the figure. Nucleotides correspond to the plus strand. Blocks were numbered right to left, to match the orientation of the gene. Haplotypes are connected by *thick lines* when they occur with a frequency greater than 10% and by *thin lines* when the frequency is ≤10%. *Numbers at the bottom* are recombination rates between adjacent blocks and are defined by a multi-allelic value of *D*'. The box and the arrow indicate the extended haplotype that confers high FN BMD. **b** LD Plot of the osteoprotegerin gene and 9 kb of its promoter, according to CEU-HapMap data. *Black or dark grey squares* indicate zones with high LD and *white or light grey squares*, zones with low LD

0.05 in the BARCOS cohort and were excluded from further analysis. All the SNPs were in Hardy–Weinberg equilibrium (HWE; p value>0.001).

Linkage disequilibrium and haplotype frequencies

Pair-wise linkage disequilibrium (*D*') and correlation coefficient (r^2) values were calculated using the Haploview software [34]. Haplotype frequencies were obtained using the HaploStats software, based on score statistics. These frequencies were corroborated with the Phase software. Haplotypes with frequencies lower than 1% were excluded.

Statistical methods

Analyses of SNPs were performed using the SPSS 12.0 statistical package. HWE was calculated by χ^2. MAFs and HWE p values for all the SNPs were calculated using the SNPATOR web tool developed by CEGEN (http://bioinformatica.cegen.upf.es/public/new_login/index.php). Analysis of covariance (when covariates are included) was used to determine the adjusted mean BMDs across genotypes and to assess the effect of each polymorphism on LS BMD and FN BMD following a General Linear Model (GLM). Height, weight, menarche age, months of breast-feeding and years since menopause were considered clinically relevant variables and included as covariates. Initially, the general model was chosen, and SNPs that showed a trend of significance were evaluated with alternative inheritance models (dominant and recessive).

Logistic regression was used to assess the effect of each polymorphism on the qualitative character fracture and crosstabs were built to determine the frequency of fractures depending on the genotype. All the analyses were corrected for the same covariates as for BMD.

The genetic effects of inferred haplotypes were analysed with R software, using the function haplo.glm of the HaploStats library [35], which follows a GLM for LS and FN BMD and a logistic regression model for fracture.

We used the Cheverud [36] proposed approach modified by Li and Ji [37] to correct for multiple testing in our

Table 2 Characteristics of the *OPG* SNPs selected for genotyping

SNPs #	rs	Position[a]	Location	LD Block[b]	alleles[c]	MAFs[d] BARCOS	MAFs[d] HapMap	HWE *p* value	Alt. name[e]
1	rs4242592	120038156	Promoter	1	G/T	0.379	0.483	0.167	
2	rs12056490	120035729	Promoter	1	A/G	**0.031**[g]	0.058	0.978	
3	rs1385504	120035514	Promoter	1	T/A	0.136	0.133	0.645	
4	rs1564861	120035090	Promoter	1	A/C	0.442	0.322	0.956	
5	rs3134069	120034169	Promoter	1	A/C	**0.048**[g]	0.067	0.678	245 T>G
6	rs2073617	120033464	5' UTR	Between1 and 2	T/C	0.433	0.442	0.904	950 T>C
7	rs2073618[f]	120033233	Exon 1	2	G/C	0.472	0.45	0.121	1181 G>C
8	rs10505346	120033024	Intron 1	2	G/T	0.240	0.192	0.705	
9	rs7463176	120026806	Intron 1	2	G/A	0.373	0.492	0.229	
10	rs11573855	120024320	Intron 1	2	A/G	**0.029**[g]	0.058	0.95	
11	rs11573856	120024176	Intron 1	2	G/A	0.108	0.067	0.61	
12	rs1485289	120023460	Intron 1	2	A/G	0.473	0.45	0.109	
13	rs3134058	120023289	Intron 1	2	G/A	0.476	0.383	0.874	
14	rs3134057	120022649	Intron 1	2	A/G	0.437	0.325	0.937	
15	rs6469788	120021931	Intron 1	3	C/A	0.380	0.44	0.016	
16	rs3134056	120021393	Intron 1	3	A/G	0.441	0.325	0.943	
17	rs1032129	120021081	Intron 1	4	A/C	0.367	0.317	0.19	
18	rs1032128	120020954	Intron 1	4	G/A	0.273	0.258	0.081	
19	rs3134054	120018292	Intron 1	5	A/G	0.441	0.325	0.958	
20	rs11573888	120017584	Intron 1	5	C/T	0.086	0.058	0.862	
21	rs4319131	120016832	Intron 1	5	A/G	0.385	0.492	0.195	
22	rs11573896	120016611	Intron 1	5	A/T	0.213	0.142	0.471	
23	rs11573906[f]	120014441	Exon 2	Between 5 and 6	G/A	**0.000**[g]	0.011	1	
24	rs6469783	120008446	Intron 3	6	C/T	0.123	0.133	0.636	

[a] Position relative to contig number NT-008046.15

[b] LD blocks of CEU-HAPMAP data according to Gabriel et al. [32]

[c] Major allele/minor allele in the "plus" strand, except for SNPs #6, #7, and #23

[d] Minor allele frequency

[e] Alternative name for the SNP in previous publications

[f] Missense substitutions in exon 1 (Lys3Asn) and exon 2 (Val104Met), respectively

[g] SNPs with MAF values below 0.05

analyses taking into account that the tests are not totally independent. According to this approach we estimated that the number of independent tests in our study is 7.03 and the corresponding multiple-test corrected *p* value is 0.0073.

Statistical power was calculated with the software Genetic Power Calculator [38]. Considering a QTL and marker allele frequency of 0.25, a linkage disequilibrium *D*'=0.80, a corrected *p* value=0.0073 and an additive genetic model, the BARCOS sample size has a 80% power to detect a genetic marker contribution of 2% in the LS BMD variance and of 3.7% in the FN BMD variance; we also have a 80% power to detect a 35% fracture risk increase associated with each risk allele.

Results

Single SNP association analysis

In the association analyses for single SNPs, most of the results with *p* values below 0.05 were found for FN BMD (eight out of the 20 analysed SNPs) either under the general or the alternative model (Table 3). These SNPs belong to five distinct LD blocks and span from the 5′ regulatory region to the intron 1 of the gene (Fig. 1). The only significant *p* value (<0.0073, see "Materials and methods") was obtained for SNP #17. Regarding LS BMD, only SNPs #17 and #18 showed a *p* value below 0.05, but none of them reached significance.

Table 3 Association between SNPs and adjusted[a] osteoporotic phenotypes: *p* values[b] for the general model (*p* values for alternative models)

SNP no.	rs	LS BMD *p* value	FN BMD *p* value	fracture *p* value
1	rs4242592	0.2	**0.036** (r: **0.023**)	**0.021**
3	rs1385504	0.417	0.077 (d: **0.031**)	0.662
4	rs1564861	0.406	0.578	0.137
6	rs2073617	0.112	0.096 (r: **0.049**)	0.089
7	rs2073618	0.33	0.182 (r: 0.065)	0.060
8	rs10505346	0.156	0.648	**0.029** (r: **0.015**)
9	rs7463176	0.224	**0.045** (r: **0.029**)	0.050
11	rs11573856	0.414	0.542	0.218
12	rs1485289	0.199	0.239	0.069
13	rs134058	0.143	0.444	0.122
14	rs3134057	0.485	0.671	0.142
15	rs6469788	0.203	0.057 (r: **0.044**)	0.066
16	rs3134056	0.517	0.64	0.143
17	rs1032129	0.091 (r: **0.041**)	***0.006*** *(d:* ***0.001****)*	0.75
18	rs1032128	**0.031** (d: **0.018**)	0.07 (d: **0.022**)	0.661
19	rs3134054	0.435	0.683	0.137
20	rs11573888	0.705	0.19	0.987
21	rs4319131	0.245	0.057 (r: **0.035**)	0.069
22	rs11573896	0.361	0.128	**0.016**
24	rs6469783	0.386	0.203	0.687

d dominant, *r* recessive, *boldface* $p<0.05$, ***boldface and italics*** $p<0.0073$ (threshold after multiple-testing correction)

[a] Adjusted for height, weight, age at menarche, breast-feeding months, and years since menopause

[b] ANCOVA for BMD, logistic regression for fractures, alternative models are displayed only if the *p* values of the general model are <0.1 and the *p* values of the alternative models are better than those of the general model

The effect sizes for both FN BMD (Table 4 (FN BMD)) and LS BMD (not shown) were explored for SNPs with $p<0.05$ and they were in the range of 0.020–0.028 g/cm^2. In five cases (SNPs #1, #6, #9, #15 and #21), the minor allele had a protective effect in homozygous state. For the other three SNPs (# 3, #17 and #18) the effects were of similar size but in the opposite direction, where the major allele was the protective one.

When fractures were analysed, three SNPs gave *p* values below 0.05, either under the general or the alternative model: SNP #1, in the 5′ regulatory region and SNPs #8 and #22, within intron 1 (Table 3). Individuals homozygous for the minor allele (T) of SNPs #8 and #22 had a 2.69- and 2.53-fold increased risk of fracture, respectively, when compared to individuals with other genotypes (Table 4 (fracture)). In contrast, individuals homozygous for the

Table 4 Effects on FN BMD and fracture of the SNPs that showed a trend of association ($p<0.05$)

FN BMD		
SNP #	Difference between means for the alternative model (95%CI)	Effect of minor allele on FN BMD or risk fracture
1	TT vs GT/GG 0.027 (0.004, 0.050)	T protective
3	TT vs TA/AA 0.021 (0.002, 0.041)	A low BMD
6	CC vs TC/TT 0.022 (0.000, 0.044)	C protective
9	AA vs GA/GG 0.027 (0.003, 0.051)	A protective
15	AA vs CA/CC 0.024 (0.001, 0.048)	A protective
17	**AA vs AC/CC 0.028 (0.011, 0.045)**	C low BMD
18	GG vs GA/AA 0.020 (0.003, 0.037)	A low BMD
21	GG vs AG/AA 0.025 (0.002, 0.048)	G protective
Fracture		
	OR (95% CI)	
1	GG/GT vs TT: 2.374 (1.113, 5.066)	T protective
8	TT vs GT/GG: 2.686 (1.224, 5.894)	T risk fracture
22	TT vs AT/AA: 2.533 (1.082, 5.929)	T risk fracture

boldface SNP with $p<0.0073$ (threshold after multiple-testing correction)

minor allele (T) of SNP #1 had a 2.37-fold decreased risk of fracture. However, these results did not stand the multiple-testing correction.

For the non-synonymous SNP rs2073618 G>C (#7), we have observed a trend for association of the C allele with higher FN BMD (p=0.065) and less risk of fracture (p=0.06).

Haplotype level association analysis

The uncorrected analysis of the effects of block-haplotypes showed several haplotypes with p values below 0.05 (Table 5). Upon correction for multiple testing, two haplotypes were associated with FN BMD and two with fracture (p values<0.0073). The AC haplotype in block 4 (containing SNPs #18 and #17 and present in approximately 27% of the chromosomes) showed a strong association with FN BMD (Table 5). For each copy of the AC haplotype, FN BMD was reduced by 0.0186 U in comparison with individuals homozygous for the major haplotype GA. The AACG haplotype in block 5 (with a frequency of 22%) was associated with FN BMD too and each copy of this haplotype reduced FN BMD by 0.0180 U as compared with the FN BMD of homozygotes for the most common haplotype. Two other haplotypes, GAAGAGTG in block 2 (including the T allele of SNP #8) and TACG in block 5 (including the T allele of SNP #22), showed significant association with fracture, where the odds ratio of each copy of each of these haplotypes was approximately 1.8 in relation to individuals homozygous for the common haplotype.

When all the data on individual SNPs and block-haplotypes was integrated, it appeared that all the alleles and haplotypes with protective effect, located in distinct blocks and with p values lower than 0.05 (shown in Tables 4 and 5), belonged to a frequent long-range haplotype (Fig. 1, arrow). This long-range haplotype had a frequency of 0.13 in the BARCOS cohort.

Discussion

Skeleton integrity requires the coordinated regulation and activity of osteoblasts (bone-forming cells) and osteoclasts (bone-resorbing cells) and an imbalance between these activities can result in severe skeletal deterioration. The *OPG* gene is a strong candidate for susceptibility to osteoporosis. While RANK/RANKL interactions lead to both differentiation and activation of osteoclasts, the interaction of OPG with RANKL negatively regulates this process. Reduced OPG levels or activity could modify the balance between bone formation and resorption, thereby producing a decrease in BMD that could lead to bone fragility and a concomitant increase in the risk of fracture. Very recent studies of genome-wide association with multiple and large sets of samples have reported that polymorphisms of the *OPG* gene contribute to determining BMD and the risk of fracture [23, 24]. These findings confirm the relevance of OPG in osteoporosis phenotypes.

The studies published so far have analysed *OPG* SNPs situated in the promoter region, 5′ UTR and the first exon. Few studies have also addressed intronic regions. The present study is the first to cover most of the gene, including 9-kb of upstream sequences, to evaluate the association between *OPG* polymorphisms and both quantitative and qualitative traits of the osteoporosis phenotype. Although the data in HapMap allowed us to cover an extensive part of the gene, the coverage was still incomplete, mainly at the 3′ part of the gene, due to lack of LD between SNPs at this region. The lack of LD structure at the 3′ end of the gene has been previously documented in

Table 5 Haplotypes associated with BMD or fracture

	Block (SNPs)	Major haplotype	Freq	Each copy of haplotype	Freq	Variation BMD	p value[a]
LS BMD	4 (18, 17)	GA	0.634	AC	0.274	−0.016	0.02100
FN BMD	1 (5–1)	ACTAG	0.449	AATAT	0.370	0.0123	0.01470
	2 (14–7)	AGGGAAGC	0.369	GAAAAGGG	0.104	−0.02041	0.00989
	3 (16, 15)	GA	0.446	AA	0.373	0.0117	0.01980
	4 (18, 17)	GA	0.634	AC	0.274	−0.0186	**0.00024**
	5 (22–19)	AGCA	0.377	AACG	0.223	−0.01803	**0.00294**
						OR (95% CI)	
Fracture	1 (5–1)	ACTAG	0.447	AATAT	0.371	0.6798 (0.4854–0.9522)	0.02500
	2 (14–7)	AGGGAAGC	0.370	GAAGAGTG	0.213	1.8022 (1.2166–2.6697)	**0.00341**
	5 (22–19)	AGCA	0.377	TACG	0.214	1.7577 (1.1944–2.5865)	**0.00436**

[a] Adjusted for height, weight, menarche age, breast-feeding months and years since menopause

Boldface p<0.0073 (threshold after multiple-testing correction)

the Chinese population [20]. On the other hand, the LD pattern in the 8q24 genomic region shows a very large block spanning from exon 2 of the *OPG* gene, in the 5′ direction, for approximately 140 kb (CEU-HapMap data).

The comparison of our data with those in the literature focused mainly on three commonly studied SNPs: 245 T>G (rs3134069, #5), 950 T>C (rs2073617, #6), and 1181 G>C (rs2073618, #7). The first SNP has been analysed by several groups [7, 8, 11], who found evidence of a negative effect on BMD or fracture risk for the minor allele (G). In our study, the low MAF obtained precluded further analysis. However, in view of the positive results of others, we checked association in the BARCOS cohort but found no significant results (data not shown). With respect to SNP #6 (950 T>C), several studies have reported no association with BMD [7, 10, 14, 19, 22]. Langdahl et al. [8] found that the rare genotype CC was associated with increased bone mass at the lumbar spine especially in osteoporotic patients. However, Yamada et al. [11] described an association between CC genotype and lower levels of proximal radius BMD in premenopausal Japanese women. For the non-synonymous SNP rs2073618 G>C, which promotes the change of the third amino acid from lysine to asparagine, several studies report a significant association with BMD or fracture. In all instances, the C allele or the CC genotype was associated with higher BMD or lower risk of fracture [16–18]. Other studies fail to find a significant association [8–10, 19, 22]. In our population, we observed a trend, in the same direction as that reported by most of the studies, but the trend was not significant.

SNP #17 (rs1032129), for which we found the C allele to be associated with lower BMD, had not been previously studied in relation to osteoporosis phenotypes. However, it was reported to be associated with susceptibility to Paget disease of bone [39]. In our study, it was the most significant SNP and the only one to stand the multiple test correction (set at $p<0.0073$, see "Materials and methods"). This SNP lies deep in intron 1, 12 kb downstream of exon 1, and its possible regulatory role was not apparent upon inspection of Genome Browser data. Whether this SNP does contribute to the phenotype or it is tagging other causative SNPs in *OPG* or within a neighbour gene, remains an open question.

Regarding haplotypes, our study revealed that four of them, belonging in blocks 2, 4 and 5, were significantly associated with bone phenotypes at $p<0.0073$. The most significant of them was the AC haplotype of block 4 (SNPs #18 and #17), which was associated with reduced FN BMD. This result matches that of SNP #17 but with improved significance ($p=0.0002$ vs. $p=0.001$), and suggests that the risk factor may be the haplotype and not the SNP alone. In block 5 (SNPs #22 through #19), two distinct haplotypes, AACG and TACG, appeared as risk factors for low FN BMD and for fracture, respectively, when compared with the homozygotes of the major haplotype AGCA. Interestingly, TACG includes all the T alleles of SNP #22 (see Fig. 1a), which in the single SNP analysis showed a trend with increased risk of fracture (Table 4 (fracture)). In this case, the improved significance of the haplotype versus the single SNP may be interpreted as follows: this particular haplotype is the one with highest risk within block 5, as compared with the major one, which may be the most protective. Still within block 5, an open question is why the minor haplotypes related to low FN BMD and to increased risk of fracture do not coincide. Finally, in block 2, haplotype GAAGAGTG was found associated with significantly increased risk of fracture when compared to the major haplotype AGGGAAGC and it included all the T alleles of SNP #8 (see Fig. 1a), which in the single SNP analysis showed a trend with increased risk of fracture (Table 4 (fracture)). One more thing, the improved significance of the haplotype analysis may be interpreted in terms of the major haplotype within block 2 being the most protective.

Taken together, the SNP and haplotype data lead to a hypothesis of a major long-range haplotype with a protective effect, where the single SNP (or combination of SNPs) responsible for the protection remains unidentified. Given the size of the LD block involved (140 kb), the causative variant(s) might lie within *OPG* or at a considerable distance 5′ of the gene. Further research would be necessary to explore this hypothesis.

Our sample size does not allow for the analysis of the interaction between genetic and environmental components, relevant for osteoporosis. Many studies have demonstrated the importance of the environmental components and the relation between them and several genes. In this respect, another limitation of our study is that information on vitamin D status, thyroid status, smoking and exercise has not been recorded. However, cases with clinically diagnosed disorder were excluded and the percentage of smokers in the Spanish females of this age is marginal (less than 10%). Finally, our study has limited power. Our sample comprised almost 1,000 individuals, which allows the detection of moderate effects of common variants. However, smaller effects or the effects of rare variants may have gone undetected.

In summary, here we report a significant association between one polymorphism and four block-haplotypes of the *OPG* gene and FN BMD and/or fracture. The challenge remains to determine whether any of the SNPs addressed here (such as #17), other undetected SNP(s) captured by the haplotypes or a combination of SNPs can explain these findings. Functional studies and efforts to replicate these results in other populations should be undertaken.

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References

1. Ralston SH (2002) Genetic control of susceptibility to osteoporosis. J Clin Endocrinol Metab 87:2460–2466
2. Liu YZ, Liu YJ, Recker RR et al (2003) Molecular studies of identification of genes for osteoporosis: the 2002 update. J Endocrinol 177:147–196
3. Hofbauer LC, Heufelder AE (2001) Role of receptor activator of nuclear factor-kappaB ligand and osteoprotegerin in bone cell biology. J Mol Med 79:243–253
4. Khosla S (2001) Minireview: the OPG/RANKL/RANK system. Endocrinology 142:5050–5055
5. Simonet WS, Lacey DL, Dunstan CR et al (1997) Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. Cell 89:309–319
6. Bucay N, Sarosi I, Dunstan CR et al (1998) osteoprotegerin-deficient mice develop early onset osteoporosis and arterial calcification. Genes Dev 12:1260–1268
7. Arko B, Prezelj J, Komel R et al (2002) Sequence variations in the osteoprotegerin gene promoter in patients with postmenopausal osteoporosis. J Clin Endocrinol Metab 87:4080–4084
8. Langdahl BL, Carstens M, Stenkjaer L et al (2002) Polymorphisms in the osteoprotegerin gene are associated with osteoporotic fractures. J Bone Miner Res 17:1245–1255
9. 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
10. 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
11. Yamada Y, Ando F, Niino N et al (2003) Association of polymorphisms of the osteoprotegerin gene with bone mineral density in Japanese women but not men. Mol Genet Metab 80:344–349
12. 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
13. Jorgensen HL, Kusk P, Madsen B et al (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
14. 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
15. 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
16. Arko B, Prezelj J, Kocijancic A et al (2005) Association of the osteoprotegerin gene polymorphisms with bone mineral density in postmenopausal women. Maturitas 51:270–279
17. 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
18. 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
19. Vidal C, Brincat M, Xuereb Anastasi A (2005) TNFRSF11B gene variants and bone mineral density in postmenopausal women in Malta. Maturitas
20. Hsu YH, Niu T, Terwedow HA et al (2006) Variation in genes involved in the RANKL/RANK/OPG bone remodeling pathway are associated with bone mineral density at different skeletal sites in men. Hum Genet 118:568–577
21. Rhee EJ, Oh KW, Jung CH et al (2006) The relationship between four single nucleotide polymorphisms in the promoter region of the osteoprotegerin gene and aortic calcification or coronary artery disease in Koreans. Clin Endocrinol (Oxf) 64:689–697
22. 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
23. Styrkarsdottir U, Halldorsson BV, Gretarsdottir S et al (2008) Multiple genetic loci for bone mineral density and fractures. N Engl J Med 358:2355–2365
24. Richards JB, Rivadeneira F, Inouye M et al (2008) Bone mineral density, osteoporosis, and osteoporotic fractures: a genome-wide association study. Lancet 371:1505–1512
25. Cardon LR, Abecasis GR (2003) Using haplotype blocks to map human complex trait loci. Trends Genet 19:135–140
26. Altshuler D, Brooks LD, Chakravarti A et al (2005) A haplotype map of the human genome. Nature 437:1299–1320
27. Morinaga T, Nakagawa N, Yasuda H et al (1998) Cloning and characterization of the gene encoding human osteoprotegerin/osteoclastogenesis-inhibitory factor. Eur J Biochem 254:685–691
28. Bustamante M, Nogues X, Agueda L et al (2007) Promoter 2–1025 T/C polymorphism in the RUNX2 gene is associated with femoral neck bmd in Spanish postmenopausal women. Calcif Tissue Int 81:327–332
29. Bustamante M, Nogues X, Mellibovsky L et al (2007) Polymorphisms in the interleukin-6 receptor gene are associated with bone mineral density and body mass index in Spanish postmenopausal women. Eur J Endocrinol 157:677–684
30. Genant HK, Wu CY, van Kuijk C et al (1993) Vertebral fracture assessment using a semiquantitative technique. J Bone Miner Res 8:1137–1148
31. Miller SA, Dykes DD, Polesky HF (1988) A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 16:1215
32. Gabriel SB, Schaffner SF, Nguyen H et al (2002) The structure of haplotype blocks in the human genome. Science 296:2225–2229
33. Tobler AR, Short S, Andersen MR et al (2005) The SNPlex genotyping system: a flexible and scalable platform for SNP genotyping. J Biomol Tech 16:398–406
34. Barrett JC, Fry B, Maller J et al (2005) Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics 21:263–265

35. Lake SL, Lyon H, Tantisira K et al (2003) Estimation and tests of haplotype-environment interaction when linkage phase is ambiguous. Hum Hered 55:56–65
36. Cheverud JM (2001) A simple correction for multiple comparisons in interval mapping genome scans. Heredity 87:52–58
37. Li J, Ji L (2005) Adjusting multiple testing in multilocus analyses using the eigenvalues of a correlation matrix. Heredity 95:221–227
38. Purcell S, Cherny SS, Sham PC (2003) Genetic Power Calculator: design of linkage and association genetic mapping studies of complex traits. Bioinformatics 19:149–150
39. Beyens G, Daroszewska A, de Freitas F et al (2007) Identification of sex-specific associations between polymorphisms of the osteoprotegerin gene, TNFRSF11B, and Paget's disease of bone. J Bone Miner Res 22:1062–1071