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Linkage and association analyses of the osteoprotegerin gene locus with human osteoporosis

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Abstract Osteoprotegerin (OPG), a secreted glycoprotein and a member of the tumor necrosis factor receptor superfamily, is considered to play an important role in the regulation of bone resorption by modifying osteoclast differentiation. Overexpression of OPG in mice has been reported to result in osteopetrosis, whereas targeted disruption of OPG in mice has been associated with osteoporosis. Accordingly, *OPG* could be a strong candidate gene for susceptibility to human osteoporosis. Here, we analyzed whether *OPG* is involved in the etiology of osteoporosis using both linkage and association analyses. We recruited 164 sib pairs in Gunma prefecture, which is located in the central part of Honshu (mainland Japan), for a linkage study, and 394 postmenopausal women in Akita prefecture, which is in the northern part of Honshu, for an association study. We identified two microsatellite polymorphisms in the linkage study, and six single-nucleotide polymorphisms (SNPs) in the *OPG* region for the association study. Although, no evidence of significant linkage between *OPG* and osteoporosis was found, a possible association of one SNP, located in the promoter region of the gene, was identified. A haplotype analysis with the six SNPs revealed that four major haplotypes account for 71% of the alleles in the Japanese population.

Key words Osteoprotegerin · Osteoporosis · Single-nucleotide polymorphism · BMD · Sib pair analysis

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Introduction

Osteoporosis is a systemic skeletal disease characterized by excessive bone resorption, typically in association with postmenopausal estrogen deficiency, which leads to low bone mass and microarchitectural deterioration with a consequent increase in bone fragility and susceptibility to fracture. Bone mineral density (BMD) is a complex trait that is influenced by multiple genes and environmental factors. Genetic factors have been estimated by twin studies to account for up to 80% of the variance in BMD (Giguere and Rousseau 2000). A number of candidate genes have been analyzed for involvement in the etiology of osteoporosis. These include, for example, the vitamin D receptor (Morrison et al. 1994), type I collagen (Grant et al. 1996), estrogen receptor (Kobayashi et al. 1996), interleukin 6 (Ota et al. 1999), and calcitonin receptor genes (Taboulet et al. 1998). Genome-wide screening of 330 DNA markers with 149 members of seven large pedigrees has been performed and several possible loci identified (Devoto et al. 1998). However, the contribution of these genes to the etiology of osteoporosis is still controversial, possibly because of racial difference, type I error, or misgenotyping (Morrison et al. 1997).

Osteoprotegerin (OPG) is a secreted glycoprotein, which was independently identified by three laboratories (Simonet et al. 1997; Tsuda et al. 1997; Tan et al. 1997) and which is considered to be a member of the tumor necrosis factor receptor superfamily. Transgenic mice that overexpress OPG exhibit a generalized increase in bone density (Simonet et al. 1997). Two separate studies using OPG-deficient mice, which showed severe early onset of osteoporosis with increased osteoclast numbers, indicated that the function of OPG is to block osteoclast formation and bone resorption (Mizuno et al. 1998; Bucay et al. 1998). Consistent with this, OPG administration protected against the decrease in bone mass that occurs in ovariectomized rats, an animal model of postmenopausal osteoporosis (Simonet et al. 1997). From these findings, *OPG* appears to be one of the most attractive candidate genes responsible

for postmenopausal-type osteoporosis susceptibility. To investigate possible effects of genetic variations at the *OPG* loci, we performed a linkage study by a sib pair analysis, and an association study with postmenopausal women by identifying single-nucleotide polymorphisms (SNPs). A possible association with one of the studied SNPs located in the promoter region of the gene was detected: individuals with TT genotype in the osteoporosis group had significantly decreased bone mineral density (BMD) when compared with those with TC or CC genotypes.

Subjects and methods

Subjects

For the sib pair analysis, DNA samples were obtained from peripheral blood of 283 Japanese women from 131 families, comprising 164 sib pairs. To determine the frequency distribution of CA repeats in Gunma Prefecture 77 unrelated Japanese women were recruited and their DNA analyzed. All lived in the area, which is in the central part of Honshu (mainland Japan). Their ages ranged from 50 to 86 years old (mean 66.2 ± 7.1 years).

For the association study, DNA samples were extracted from peripheral blood of 394 postmenopausal Japanese women ranging in age from 66 to 92 (mean 73.2 ± 5.8 years) living in Akita prefecture, which is in the northern part of Honshu. No participant in either study group had medical complications or was undergoing treatment for conditions known to affect bone metabolism, such as pituitary disease, hyperthyroidism, primary hyperparathyroidism, renal failure, adrenal disease, or collagen disease, and none was receiving estrogen replacement therapy. To analyze the haplotype pattern of *OPG* in the Japanese population, 48 DNA samples from volunteers of both sexes ranging in age from 20 to 22, recruited in our medical school, were used. The ethics committee of the Asahikawa Medical College approved the protocol of this study. The nature, purpose, and potential risks of the study were carefully explained to all patients before they agreed to participate. All were volunteers and gave informed consent prior to the study.

Measurement of bone mineral density (BMD)

Instruments for BMD measurements were different between Gunma and Akita prefectures because we used the installed machine in each health check-up center. The BMD of the radial bone (expressed in g/cm^2) of each participant was measured by means of dual energy X-ray absorptiometry by using a DTX-200 (Osteometer MediTech, Hawthorne, CA, USA) instrument in Gunma, and with a DPX-L (Lunar Madison, WI, USA) instrument in Akita. In the sib pair linkage analysis, osteoporosis and osteopenia were defined as a decrease in BMD below 70% and 80%, respectively, of the mean in young adult women (cut-off values 0.333 = −2.6SD and 0.381 = −1.8SD), according to the general criteria recommended by the Japanese Society for Bone and Mineral Research (Orimo et al. 1996). On the other hand, in the association study with the Akita population, the analysis was performed on the quantitative phenotype, BMD. Consequently, factors (age, height, and weight) that would affect BMD values had to be adjusted. Thus, we used the following formula: body mass index (BMI) = body weight (kg)/body height^2 (m); adjusted BMD (adjBMD) = BMD − 0.0052 × (73.2 − age) + 0.0088 × (23.2 − BMI) (Ota et al. 2001).

Genotyping of microsatellite polymorphisms

A human genomic clone containing the *OPG* gene was identified by a P1-derived chromosome (PAC) Human Genomic polymerase chain reaction (PCR) Screening Kit (Incyte Genomics, Palo Alto, CA, USA), by using primer sequences derived from the 3′ portion of the gene. A fragment containing the CA repeat was identified by Southern blotting of PAC DNA digested by *Hae*III or *Sau*3AI with a $(GT)_{20}$ oligonucleotide probe, and then subcloned and sequenced. Two informative repeat sequences, named OPG1 and OPG2 and shown in Fig. 1, were identified. PCR primers were designed to flank the repeat sequences for the polymorphism analysis (Fig. 1). The PCR primers used were OPG1F (forward), 5′-GCACACACGCTCTGTTTC TC-3′; OPG1R (reverse), 5′-GGAGGGTGGTAACTTG GGAT-3′; OPG2F (forward), 5′-AGTCTGGGCAACA GAGCAAG-3′; and OPG2R (reverse), 5′-CTAGCCTGA TGAATTGTCATC-3′. Fluorescent-labeled primers were used for genotyping. PCR amplification was carried out as described below for genotyping except that the annealing temperature was 55°C. Electrophoresis was performed with an ABI 377 DNA sequencer; the data were extracted by using GeneScan Analysis software and analyzed by the Genotyper program (Applied Biosystems, Foster City, CA, USA).

a. OPG1

GCACACACGCTCTGTTTCTCTCTCTCTCTGTCTGTCTCTCT
CTCTGTGCATGTGAGTGCTT**TGTGTGTGTGTGTGTGTGTGT**
GTGTGTGTGTGTGTGGAATCAATATAGTAATAAGATATTTA
AAATTGTTAAATCCCAAGTTACCACCCTCC

b. OPG2

AGTCTGGGCAACAGAGCAAGATTTCAT**CACACACACACA**
CACACACACACACACACACATTAGAAATGTGTACTTGGC
TTTGTTACCTATGGTATTAGTGCATCTATTGCATGGAAC
TTCCAAGCTACTCTGGTTGTGTTAAGCTCTTCATTGGGT
ACAGGTCACTAGTATTAAGTTCAGGTTATTCGGATGCAT
TCCACGGTAGTGATGACAATTCATCAGGCTAG

Fig. 1a,b. Nucleotide sequences of the polymorphic repeats and their flanking region in the osteoprotegerin (*OPG*) gene locus. Sequences used for forward and reverse primers are *underlined*. CA (or GT) repeats are shown in *bold*. **a** *OPG1*. **b** *OPG2*

Search for single-nucleotide polymorphisms (SNPs)

A total of 23 primer sets were designed to amplify 12 kb of the *OPG* gene containing 1100 bases of the promoter region, all five exons and introns, and 840 base pairs of the 3′ flanking region. SNPs were detected by sequencing DNA samples from ten independent volunteers (20 alleles) on an ABI 310 sequencer (Applied Biosystems).

Genotyping of detected SNPs

Primers for each SNP were designed for the amplification refractory modification system (ARMS) technique (Newton et al. 1989). In all, six SNPs were analyzed: one in the 5′ untranslated region (5′UTR) (SNP1), one in exon 1 (SNP2), two in intron 2 (SNP3 and SNP4), and two in intron 3 (SNP5 and SNP6). ARMS primers were designed to amplify a region of about 250 bp. Sequences of the primers used are as follows: OPG5UTRA (forward), 5′-GGCTGC GGAGACGCACCCGCA-3′; OPG5UTRC (forward), 5′-GGCTGCGGAGACGCACCCGCC-3′; OPG5UTRAS (reverse), 5′-AGCATGGCATAACTTGAAAGC-3′; OPGE1K (forward), 5′-CGGGGACCACAATGAAC TAG-3′; OPGE1N (forward), 5′-CGGGGACCACAATG AACTAC-3′; OPGE1AS (reverse), 5′-GCTGTCTTCCA TAAAGTCAGC-3′; OPGi21C (forward), 5′-ATGCTAG AGTTTTGTGCATC-3′; OPGi21T (forward), 5′-ATG CTAGAGTTTTGTGCATT-3′; OPGi21AS (reverse), 5′-TTTCCTTTCTGAGTTAGCAGG-3′; OPGi22C (forward), 5′-ACTAAATTGCTTGGTATTTGCC-3′; OPGi22T (forward), 5′-ACTAAATTGCTTGGTATTTG CT-3′; OPGi22AS (reverse), 5′-TACAAAATCGTACAA AGACGT-3′; OPGi31G (forward), 5′-TCTCCCCAAAC AGTTTTGCG-3′; OPGi31A (forward), 5′-TCTCCCCAA ACAGTTTTGCA-3′; OPGi31AS (reverse), 5′-GTGCA CAATAAATGAAAAAAAGT-3′; OPGi32T (forward), 5′-CAGTTCCAGCATTGTTTAAT-3′; OPGi32C (forward), 5′-CAGTTCCAGCATTGTTTAAC-3′; and OPGi32AS (reverse), 5′-CTACTACCTATATTCATCT GA-3′. To confirm the reaction, a part of the β-globin gene was amplified as well, as a positive control. The PCR primers used were BGLOS (forward), 5′-ACACAACTGTG TTCACTAG-3′ and BGLOAS (reverse), 5′-CATGAGC CTTCACCTTAGGG-3′, which amplified a 360-bp region. After amplification, 3% agarose gel or 12% acrylamide gel electrophoresis were performed for genotyping. For some of the SNPs, the PCR products were sequenced to confirm the results obtained by the ARMS method. The PCR primers for sequencing were as follows: for SNP1 and SNP2, OPGSNP1F (forward), 5′-GCTCTCCCAGGGGACAGA CA-3′ and OPGSNP1R (reverse), 5′-AGACCAGGTGGC AGCAGCCT-3′; for SNP3 and SNP4, OPGSNP2F (forward), 5′-TAGCGTCTTTAGTTGTGGACT-3′ and OPGSNP2R (reverse), 5′-CCGGAACATATGTTGTCG TG-3′; and for SNP5 and SNP6, OPGSNP3F (forward), 5′-GTGTTAAGCTCTTCATTGGGTA-3′ and OPGSNP3R (reverse), 5′-AAATGGGAGTAATGGGTGTTTG-3′. PCR was performed in a volume of 12.5 μl containing 20 ng genomic DNA, 10 mM Tris HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 200 μM deoxyribonucleotide triphosphates (dNTPs), 10 pmol of each primer, and 0.25 units of *Taq* polymerase. PCR amplification was performed with 30 cycles of 94°C for 30 s, 49°–62°C for 30 s, and 72°C for 30 s, depending on the region analyzed, with a final extension step of 5 min at 72°C in a Gene Amp PCR9600 System (Applied Biosystems). The amplified mixture was electophoresed in 1.5% agarose gel to isolate the fragment containing the PCR product. After that, the PCR product was extracted by using a Geneclean III Kit (Bio 101, Vista, CA, USA). The sequencing reaction and electrophoresis were performed with a BigDye Terminator kit (Applied Biosystems) following the manufacturer's protocol.

Statistical analysis

In an analysis using osteoporosis and osteopenia as the affected status, we analyzed three classes of sib pairs: (1) both sib unaffected (clinically concordant unaffected sib pairs); (2) one sib affected and the other not (clinically discordant sib pairs); and (3) both sibs affected (clinically concordant affected sib pairs). A nonparametric linkage analysis was performed by using the SIBPAL program (version 2.7) of the SAGE package (Case Western Reserve University, Cleveland, OH, USA). A significant increase in allele sharing (>0.5) for concordant pairs and/or a significant decrease in allele sharing (<0.5) for discordant pairs was considered evidence for linkage. Here, the term "allele sharing" means the proportion of shared alleles in a sib; thus, the value ranges from 0 to 1 and the expected value is 0.5.

For the association analysis, we compared BMD and adjBMD as a quantitative phenotype between genotype groups (TT vs. TC+CC and TT+TC vs. CC in SNP1; GG vs. GC+CC and GG+GC vs. CC in SNP2) by using both *t*-test (parametric) and the Mann-Whitney U test (nonparametric) from the statistical analysis system package (SAS Institute, Cary, NC, USA). A P value of < 0.05 was considered statistically significant.

Haplotype analysis

Haplotype frequency was estimated by the maximum-likelihood method by using a simplified version of the computer program GENEF (J-M Lalouel, University of Utah, unpublished). Procedures to generate the haplotype, are described in detail in Jeunemaitre et al. (1997). Briefly, two SNPs were chosen to generate a haplotype, followed by sequential inclusion of one SNP at a time. All haplotypes below a frequency of 1/4 N, where N is the sample size, were automatically eliminated.

The strength of linkage disequilibrium (LD) was calculated by using Arlequin software for population genetic data analysis (http://anthropologie.unige.ch/arlequin). Pairwise LD was estimated as $D = x_{ij}\text{-}p_i p_j$, where x_{ij} is the frequency of haplotype A_1B_1, and p_1 and p_2 are the frequencies of alleles A_1 and B_1 at loci A and B, respectively. A

standardized LD coefficient, r, is given by $D/(p_1p_2q_1q_2)^{1/2}$, where q_1 and q_2 are the frequencies of the other alleles at loci A and B, respectively (Hill and Robertson 1968). Lewontin's coefficient D′ is given by D/D_{max}, where $D_{max} = \min[p_1p_2,q_1q_2]$ when $D < 0$ and $D_{max} = \min[q_1p_2,p_1q_2]$ when $D > 0$ (Lewontin 1984). An appropriate LD measure for association studies, d, is given by $d = D/p_1q_2$, where p_1 is the variant frequency and p_2 is the marker allele frequency (Kruglyak 1999).

Results

Sib pair linkage analysis

The frequency distribution of two microsatellite polymorphisms, OPG1 and OPG2, in the Gunma area was determined by using a randomly selected group of 77 unrelated Japanese women (Table 1). The polymorphic PCR products of OPG1 and OPG2 contained 16–23 and 15–21 repeats, respectively. The frequency of heterozygotes was calculated as 80.1% for OPG1 and 53.7% for OPG2. The total number of successfully genotyped sib pairs for the linkage analysis was 164 for OPG1 and 153 for OPG2. The results of the analysis for osteoporosis and osteopenia are shown in Table 2. No significant linkage of OPG1 or OPG2 to either osteoporosis or osteopenia was observed.

Table 1. Frequency distribution of CA repeat alleles in the *OPG* gene locus among 77 Japanese women

	OPG1		OPG2	
Alleles	Repeat no.	Freq.	Repeat no.	Freq.
A1	16	0.0001	15	0.0001
A2	17	0.2208	16	0.6474
A3	18	0.2662	17	0.1282
A4	19	0.2078	18	0.0641
A5	20	0.1169	19	0.1538
A6	21	0.1429	20	0.0000
A7	23	0.0455	21	0.0064

OPG, osteoprotegerin

Association analysis

A total of six SNPs (SNP1 to SNP6, see Subjects and methods) were detected in the *OPG* gene locus. The observed number of genotypes in each SNP site did not differ significantly from what would be expected from the Hardy-Weinberg equilibrium. Two SNPs (SNP1 and SNP2) out of six, located in the 5′-UTR and in exon 1, appeared to be more interesting in terms of OPG function. SNP1, either T or C at 223 bp upstream from the translation initiation site, could have some influence on promoter activity. SNP2, either G or C at the 9th base of the signal peptide coding sequence, changed the third amino acid from lysine to asparagine. Because of their possible role in OPG function and the limited amount of available DNA, we decided to genotype these two SNPs for the 394 samples from postmenopausal women of Akita Prefecture. Age, height, weight, BMI, BMD, and adjusted BMD in each genotype group are shown in Table 3 as mean ± SD. The analysis was performed with BMD and adjBMD as a quantitative phenotype between genotype groups (see Subjects and methods). In SNP1, individuals with the TT genotype showed significantly low BMD and adjBMD when compared with those of the TC or CC genotypes (Table 4) ($P = 0.028$ in BMD and $P = 0.021$ in adjBMD, by the Mann-Whitney U-test). Because our samples showed a nearly normal distribution, a *t*-test was also used, and it also indicated a significant difference ($P = 0.023$). This result may indicate that the allele with C in SNP1 has a protective effect with respect to osteoporosis. On the other hand, in SNP2, no significant result was obtained ($P = 0.561$ for BMD and $P = 0.369$ for adjBMD by the Mann-Whitney U-test; $P = 0.242$ by the *t*-test when GG was compared with GC+CC).

Table 2. Sib pair linkage

Locus	Status	Pairs	Mean	SD	SE	*t*-values	*P*-values
Osteoporosis							
OPG1	0	82	0.517	0.262	0.029	0.591	0.28
	1	49	0.470	0.259	0.037	0.807	0.21
	2	33	0.499	0.277	0.048	−0.020	0.51
OPG2	0	77	0.492	0.205	0.023	−0.324	0.63
	1	45	0.448	0.221	0.033	1.567	0.06
	2	31	0.447	0.185	0.033	−1.593	0.94
Osteopenia							
OPG1	0	44	0.516	0.252	0.038	0.425	0.34
	1	59	0.491	0.271	0.035	0.244	0.40
	2	61	0.495	0.268	0.034	−0.140	0.56
OPG2	0	41	0.483	0.216	0.034	−0.501	0.69
	1	57	0.492	0.200	0.026	0.313	0.38
	2	55	0.438	0.205	0.028	−2.231	0.99

Status: 0, concordant unaffected pairs; 1, discordant pairs; 2, concordant affected pairs. Criteria for diagnosis were bone mineral density (BMD) <70% (osteoporosis) and <80% (osteopenia) of the mean among young adult females. Mean is the average value of allele sharing. SD and SE are standard deviation and standard error, respectively, of the value of allele sharing

Table 3. Polymorphic status and clinical characteristics

	SNP1			SNP2		
	Genotype TT	TC	CC	Genotype GG	GC	CC
n	171	168	55	203	159	32
Age (years)	73.2 ± 6.1	73.1 ± 5.7	73.1 ± 5.9	73.0 ± 6.1	73.1 ± 5.7	74.2 ± 5.2
Height (cm)	144.7 ± 6.1	144.6 ± 5.9	145.4 ± 6.4	144.6 ± 6.2	144.4 ± 5.8	145.5 ± 6.0
Weight (kg)	49.1 ± 8.0	49.0 ± 8.2	50.3 ± 8.7	49.4 ± 8.0	48.9 ± 8.6	50.0 ± 7.7
BMI	23.4 ± 3.4	23.0 ± 3.2	23.4 ± 3.4	23.2 ± 3.4	23.0 ± 3.3	23.3 ± 3.5
BMD (g/cm^2)	0.296 ± 0.075	0.309 ± 0.075	0.314 ± 0.070	0.303 ± 0.074	0.307 ± 0.077	0.297 ± 0.059
adjBMD (g/cm^2)	0.297 ± 0.060	0.311 ± 0.058	0.312 ± 0.060	0.303 ± 0.058	0.308 ± 0.061	0.302 ± 0.060

BMI, body mass index; adjBMD, adjusted bone mineral density; SNP, single-nucleotide polymorphism

Table 4. Comparison of adjBMD between SNP1 genotype groups

	SNP1 genotype	
	TT	TC+CC
n	171	223
Age (years)	73.2 ± 6.1	73.1 ± 5.7
Height (cm)	144.7 ± 6.1	144.8 ± 6.0
Weight (kg)	49.1 ± 8.0	49.3 ± 8.3
BMI	23.4 ± 3.4	23.4 ± 3.3
BMD (g/cm^2)	0.296 ± 0.075*	0.310 ± 0.074
adjBMD (g/cm^2)	0.297 ± 0.060**	0.311 ± 0.059

Values are mean ± SD
* $P = 0.028$, ** $P = 0.021$ (Mann-Whitney U test, TT versus TC+CC)

Haplotype analysis

In order to find the OPG susceptibility haplotype for osteoporosis or other diseases in the future, a haplotype analysis was performed with all six SNPs, based on 37–48 Japanese DNA samples. Unfortunately, the amount of DNA collected from the 394 postmenopausal women of Akita Prefecture was not enough for genotyping these four additional SNPs. Thus, a haplotype association study could not be performed in the present study. Genotype and allele frequencies of these SNPs are shown in Table 5. As shown in Table 6, a strong linkage disequilibrium of variable degree was observed among these SNPs. Haplotype construction with these SNPs revealed that four major haplotypes accounted for 71% of the population (Table 7).

Discussion

In the current study, we investigated the role of OPG in the pathogenesis of osteoporosis by both linkage and association analyses. In the sib pair linkage analysis, two disease criteria (osteoporosis and osteopenia) were used to classify sib pairs. As shown in Table 2, even including osteopenia, it was possible to recruit only 61 affected sib pairs for OPG1 and 55 for OPG2. If λ_s is 3.0, a power to detect an effect of about 80% can be attained with 100 affected pairs (Risch 1990). However, the role of OPG in the pathogenesis of osteoporosis or osteopenia is not estimated to be very strong (Giguere and Rousseau 2000). Thus, the negative result of the present linkage analysis may be because the sample size was too small for detection, even if OPG has some role in the pathogenesis of osteoporosis.

In the association study, we analyzed the quantitative phenotype itself, BMD and adjBMD, which is considered a more powerful technique than a comparison between a disease group and a control group classified according to a quantitative variable (Duggirala et al. 1997). When a group of TT genotype in SNP1 was compared with a group of TC or CC genotype, significantly lower BMD and adjBMD values were identified by both parametric and nonparametric tests (Table 4), with marginal *P* values. SNP1, a T to C change 233 bp upstream from the translation initiation site, was located in the promoter region. Thus, this polymorphism could derive its functional significance by altering the level of promoter activity. A promoter assay with a reporter gene fusion construct could clarify this point. SNP2 changed the third amino acid (lysine to asparagine) of the signal peptide, which is necessary for OPG to be secreted from the cell. Lysine is a basic amino acid, while asparagine is an uncharged polar amino acid. In angiotensinogen, another secreted protein, a basic amino acid in the signal peptide was shown to drastically affect secretory kinetics (Nakajima et al. 1999a). Therefore, although we could not detect a significant disease association with SNP2, the point mutation could also influence OPG's secretory kinetics.

An association study with a haplotype analysis is a powerful tool for determining a genetic contribution to a common disease. Our analysis of the *OPG* gene revealed that four major haplotypes account for 71% of the population as reflected by the considerable linkage disequilibrium among the six SNPs (Table 6). With this information, another association analysis could be performed in the future with a larger sample size to have a reasonable power to detect an effect. Moreover, our finding of the haplotype profile of the gene is useful information for the study of the role of OPG in other disease conditions. In fact, since OPG was identified as a novel secreted protein involved in the regulation of bone density in 1997 (Simonet et al. 1997), several findings suggesting the relevance of OPG to other conditions have been reported. First, OPG-deficient mice had arterial calcification in the large arteries by 2 weeks of age (Bucay et al. 1998), indicating a possible role of OPG in diseases showing

Table 5. Genotype and allele frequencies of all six SNPs

Polymorphism	Nucleotide position	Genotype			No.	Allele frequency	
SNP1	−223[a]	TT	TC	CC		T	C
		18	22	8	48	0.6	0.4
SNP2	+9[a]	GG	GC	CC		G	C
		27	17	4	48	0.74	0.26
SNP3	IVS2-749G>T[b]	TT	TG	GG		T	G
		35	10	1	46	0.87	0.13
SNP4	IVS2-5C>T[b]	CC	CT	TT		C	T
		29	8	0	37	0.89	0.11
SNP5	IVS3-1059G>A[b]	GG	GA	AA		G	A
		35	9	2	46	0.86	0.14
SNP6	IVS3-915T>C[b]	TT	TC	CC		T	C
		16	21	8	45	0.59	0.41

[a] Nucleotide position is identified from the translation initiation site
[b] SNPs designated according to the mutation nomenclature in den Dunnen and Antonarakis (2000)

Table 6. Strength of linkage disequilibrium

Polymorphism		SNP1	SNP2	SNP3	SNP4	SNP5
SNP2	D′	0.90	...	...	...	...
	r^2	0.42				
SNP3		−0.65	−0.64	...	...	...
		0.06	0.03			
SNP4		0.53	−1	−1	...	...
		0.05	0.05	0.03		
SNP5		−0.78	−1	−1	−0.21	...
		0.09	0.08	0.04	0	
SNP6		0.81	1	1	−0.03	−1
		0.30	0.24	0.13	0	0.33

Table 7. Haplotype analysis of the *OPG* gene

SNP1 T→C	SNP2 G→C	SNP3 T→G	SNP4 C→T	SNP5 G→A	SNP6 T→C	Frequency
C	C	T	C	G	T	0.249
T	G	T	C	G	C	0.174
T	G	T	C	A	C	0.160
T	G	G	C	G	T	0.123
T	G	T	C	G	T	0.084
C	G	T	T	G	T	0.069
C	G	T	C	G	T	0.039
T	G	T	T	G	C	0.032
C	G	G	C	G	T	0.023
C	G	T	T	A	C	0.016
C	G	T	C	G	C	0.015
T	C	G	C	G	T	0.015
					Total	1.000

arterial calcification. In addition, an association of serum OPG levels with diabetes and cardiovascular mortality suggests the possibility that OPG may be a cause of vascular calcification (Browner et al. 2001). Second, expression of OPG is not restricted to bone; it is expressed in a variety of tissues and cell systems, such as heart, lung, kidney, placenta, liver, thyroid gland, spinal cord, and brain. In addition, it is expressed in various immune and hematological tissues and mesenchymal organs (Hofbauer 1999). Third, a possible role of OPG in the immune system was shown in experiments with OPG-deficient mice by Yun et al. (2001), who reported that OPG regulates B cell maturation and development.

Ethnic differences in the genetic background of diseases are sometimes observed. For example, although Grant et al. (1996) reported the association of osteoporosis with a polymorphic SP1-binding site in the collagen type Iα1 gene, we could not find the polymorphism in a study of Japanese individuals (Nakajima et al. 1999b). Thus, similar OPG studies should be performed with samples from other ethnic groups because OPG might also be related to osteoporosis in populations other than Japanese.

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