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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 TNF receptor superfamily, is considered to play an important role in the regulation of bone resorption through 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 of 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 for a linkage study, and six single nucleotide polymorphisms (SNPs) for an association study in the OPG region. Although, no evidence of significant linkage between OPG and osteoporosis was found, a possible association of one SNP, which locates in the promoter region of the gene, was identified. Haplotype analysis with 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

Introduction

Osteoporosis is a systemic skeletal disease characterized by excessive bone resorption, typically in association with postmenopausal estrogen deficiency, and leads to low bone mass and micro-architectural 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 are 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, 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 J et al. 1998). Genome-wide screening of 330 DNA markers with 149 members of seven large pedigrees has been performed, identifying several possible loci (Devoto et al. 1998). However, the contribution of these genes to the etiology of osteoporosis is still controversial, possibly because of a racial difference, type I error, mis-genotyping (Morrison et al. 1997), and so on.

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 is considered to be a member of the tumor necrosis factor receptor superfamily. Transgenic mice that overexpressed 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). With these findings, OPG appeared 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 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, which locates in the promoter region of the gene, was detected: individuals with TT genotype in osteoporosis group have significantly decreased bone mineral density (BMD) when compared with those of TC or CC genotypes.

Materials and Methods

Subjects

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

For an association study, DNA samples were extracted from peripheral blood of 394 postmenopausal Japanese women living in Akita prefecture, which is northern part of Honshu, whose ages ranged from 66 to 92 (mean 73.2 ±5.8 years). No participant in both study groups 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 haplotype pattern of OPG in the Japanese population, 48 DNA samples derived from volunteers of both sex, recruited in our medical school, whose ages ranged from 20 to 22, 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 are different between Gunma and Akita, because we used installed machine in each health check-up center. BMD of the radial bone (expressed in g/cm^2) of each participant was measured by means of dual energy X-ray absorptiometry using a DTX-200 (Osteometer MediTec, USA) in Gunma, and a DPX-L (Lunar Co., USA) in Akita. In sib pair linkage analysis, criteria for the diagnosis of osteoporosis and osteopenia was defined as a decrease of 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 general criteria recommended by the Japanese Society for Bone and Mineral Research (Orimo et al. 1996). On the other hand, in an association study with Akita population, the analysis was performed on quantitative phenotype, BMD. For this purpose, factors (age, height, and weight), those would affect BMD values, have to be adjusted. Thus, we used 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 polymorphism

A human genomic clone containing the OPG gene was identified by a P1 derived

chromosome (PAC) Human Genomic PCR Screening Kit (Incyte Genomics, USA), 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 $(GT)_{20}$ oligonucleotide probe, then subcloned and sequenced. Two informative repeat sequences, named OPG1 and OPG2, shown in Fig. 1 were identified. PCR primers were designed to flank the repeat sequences for polymorphism analysis (Fig.1). The PCR primers used were: OPG1F (forward): 5'-GC-ACACACGCTCTGTTTCTC-3', OPG1R (reverse): 5'-GGAGGGTGGTAACTTGGG-AT-3', OPG2F (forward): 5'-AGTCTGGGCAACAGAGCAAG-3', OPG2R (reverse): 5'-CTAGCCTGATGAATTGTCATC-3'. Fluorescent-labeled primers were used for genotyping. PCR amplification was carried out as described below for genotyping except the annealing temperature was 55°C. Electrophoresis was performed with an ABI 377 DNA sequencer; the data were extracted by GeneScan Analysis software and analyzed by the Genotyper program (Applied Biosystems, USA).

Search of single nucleotide polymorphisms (SNPs)

A total of 23 primer sets were designed to amplify 12 kb of the OPG gene, that contains 1,100 bases of promoter region, all five exons and introns, and 840 base pairs of 3' flanking region. SNPs were detected by sequencing DNA samples derived from ten independent volunteers (20 alleles) on an ABI 310 sequencer (Applied Biosystems,

USA).

Genotyping of detected SNPs

Primers for each SNP were designed for the amplification refractory modification system (ARMS) technique (Newton et al, 1989). Total 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 are as follows; OPG5UTRA (forward): 5'-GGCTGCGGAGACGCACCCGCA-3', OPG5UTRC (forward): 5'-GGCTGCGGAGACGCACCCGCC-3', OPG5UTRAS (reverse): 5'-AGCATGGCATAACTTGAAAGC-3', OPGE1K (forward): 5'-CGGGGACCACAA-TGAACTAG-3', OPGE1N (forward): 5'-CGGGGACCACAATGAACTAC-3', OPGE1AS (reverse): 5'-GCTGTCTTCCATAAAGTCAGC-3', OPGi21C (forward): 5'-ATGCTAGAGTTTTGTGCATC-3', OPGi21T (forward): 5'-ATGCTAGAGTTTTG-TGCATT-3', OPGi21AS (reverse): 5'-TTTCCTTTCTGAGTTAGCAGG-3', OPGi22C (forward): 5'-ACTAAATTGCTTGGTATTTGCC-3', OPGi22T (forward): 5'-ACTAA-ATTGCTTGGTATTTGCT-3', OPGi22AS (reverse): 5'-TACAAAATCGTACAAAGA-CGT-3', OPGi31G (forward): 5'-TCTCCCCAAACAGTTTTGCG-3', OPGi31A (forward): 5'-TCTCCCCAAACAGTTTTGCA-3', OPGi31AS (reverse): 5'-GTGCAC-AATAAATGAAAAAAAGT-3', OPGi32T (forward): 5'-CAGTTCCAGCATTGTTTA-

AT-3', OPGi32C (forward): 5'-CAGTTCCAGCATTGTTTAAC-3' and OPGi32AS (reverse): 5'-CTACTACCTATATTCATCTGA-3'. To confirm the reaction, a part of the β-globin gene was amplified together as a positive control. The PCR primers used were: BGLOS (forward): 5'- ACACAACTGTGTTCACTAG-3', BGLOAS (reverse): 5'-CATGAGCCTTCACCTTAGGG-3', which amplifies a 360bp gene region. After amplification, 3% agarose gel or 12% acrylamide gel electrophoresis were performed for genotyping. In some of the SNPs, PCR products were sequenced to confirm the results obtained by the ARMS method. The PCR primers for sequencing are as follows; for SNP1 and SNP2: OPGSNP1F (forward): 5'- GCTCTCCCAGGGGACAG-ACA-3', OPGSNP1R (reverse): 5'-AGACCAGGTGGCAGCAGCCT-3', for SNP3 and SNP4, OPGSNP2F (forward): 5'- TAGCGTCTTTAGTTGTGGACT-3', OPGSNP2R (reverse): 5'-CCGGAACATATGTTGTCGTG-3', for SNP5 and SNP6, OPGSNP3F (forward): 5'-GTGTTAAGCTCTTCATTGGGTA-3', OPGSNP3R (reverse): 5'-AAAT-GGGAGTAATGGGTGTTTG-3'. PCR was performed in a volume of 12.5μl containing 20 ng genomic DNA, 10mM Tris HCl (pH 8.4), 50mM KCl, 1.5mM $MgCl_2$, 0.01% gelatin, 200μM deoxyribonucleotide triphosphate (dNTP)s, 10pmol of each primer, and 0.25 units of Taq polymerase. PCR amplification was performed through 30 cycles of 94°C for 30sec, 49-62°C for 30 sec, and 72°C for 30 sec, depending on the region analyzed, with a final extension step of 5 min at 72°C in a Gene Amp PCR9600 System (Applied Biosystems, USA). 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 Geneclean III Kit (Bio 101, USA). Using BigDye Terminator kit (Applied Biosystems, USA), sequencing reaction and electrophoresis were performed following the manufacture's protocol.

Statistical analysis

In an analysis using osteoporosis and osteopenia as the affected status, we analyzed three classes of sib pairs; (1) both sibs unaffected (clinically concordant unaffected sibs pairs); (2) one sib affected and the other not (clinically discordant sib pairs); and (3) both sibs affected (clinically concordant affected sib pairs). Non-parametric linkage analysis was performed using the SIBPAL program (version 2.7) of the SAGE package (Case Western Reserve University, 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 expected value is 0.5.

For an 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 both *t*-test (parametric) and Mann-Whitney U test (non-parametric) using a statistical analysis system package (SAS

Institute Inc., Cary, NC). A *P* value of <0.05 was considered statistically significant.

Haplotype analysis

Estimation of haplotype frequency was performed by the maximum-likelihood method 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 1/4N, where N is the sample size, were automatically eliminated.

Strength of linkage disequilibrium (LD) was calculated using Arlequin program, software for population genetic data analysis (Schneider et al. 2000), which is available at http://anthropologie.unige.ch/arlequin. Pair-wise LD was estimated as $D=x_{ij}-p_ip_j$, 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 $D<0$ or $D_{max}=\min[q_1p_2,p_1q_2]$ when $D>0$ (Lewontin 1984). And 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

Frequency distribution of two microsatellite polymorphisms, OPG1 and OPG2, in Gunma area was determined with randomly selected unrelated 77 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% in OPG1 and 53.7% in OPG2. The total number of successfully genotyped sib pairs for linkage analysis was 164 in OPG1 and 153 in OPG2. The results of the analysis for osteoporosis and osteopenia are shown in Table 2. No significant linkage of the OPG1 and OPG2 to both osteoporosis and osteopenia was observed.

Association analysis

A total of six SNPs (SNP1 to SNP6, see materials and methods) were detected in the OPG gene locus. The observed number of genotypes in each SNP site did not differ significantly from those that would be expected from the Hardy-Weinberg equilibrium. Two SNPs (SNP1 and SNP2) out of six, located in the 5'-UTR and 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 the possible role on OPG

function and limited amount of DNA, we decided to genotype these two SNPs with 394 samples derived from postmenopausal women in Akita. Age, height, weight, BMI, BMD and adjusted BMD in each genotype group were shown in Table 3 presented as mean ± SD. Analysis was performed with BMD and adjBMD as a quantitative phenotype between genotype groups (see materials and methods). In SNP1, individuals of TT genotype showed significantly low BMD and adjBMD when compared with those of TC or CC genotypes (Table 4) (P=0.028 in BMD and P=0.021 in adjBMD by Mann-Whitney U-test). As normal distribution of our samples was almost, but not satisfactorily, ascertained, *t*-test was also employed to analyze, indicating a significant difference (P=0.023). This result may indicate that the allele with C in SNP1 has a protective effect on osteoporosis. On the other hand, in SNP2, no significant result can be obtained with the analysis (P=0.561 in BMD and P=0.369 in adjBMD by Mann-Whitney U-test, P=0.242 by *t*-test when compared GG versus GC+CC).

Haplotype analysis

In order to find susceptibility haplotype of OPG for osteoporosis or other diseases in the future, haplotype analysis was performed with all six SNPs based on 37 to 48 Japanese DNA samples. Unfortunately, DNA amount of 394 samples derived from postmeno-pausal women in Akita was not enough for these additional four SNPs genotyping.

Thus, 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, strong linkage disequilibrium with 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 sib pair linkage analysis, two criteria of disease (osteoporosis and osteopenia) were used to classify sib pairs. As shown in Table 2, even with definition of osteopenia, only 61 affected sib pairs in OPG1 and 55 in OPG2 have been able to be recruited. If λs is 3.0, about 80% of power to detect an effect can be attained with 100 affected pairs (Risch 1990). However, contribution of OPG in the pathogenesis of osteoporosis or osteopenia is estimated not to be that strong (Giguere and Rousseau 2000). Thus, the negative result of present linkage analysis can be due to a small sample size for detection, even when OPG have some role in the pathogenesis.

In an association study, we analyzed the quantitative phenotype itself, BMD and adjBMD, as it is reported to be more powerful than comparison between disease group and control group classified according to a quantitative variable (Duggirala et al, 1997). When compared a group of TT genotype in SNP1 versus a group of TC or CC genotype, a significantly lower BMD and adjBMD value was identified by both parametric and non-parametric test (Table 4) with marginal *P* value. SNP1, a T to C change at 233 bp upstream from the translation initiation site, is located in the promoter region. Thus, this polymorphism could have a functional significance by altered level of promoter activity. Promoter assay with a reporter gene fusion construct could

clarify this point. SNP2 changes 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 significant association with SNP2, the point mutation could also have an influence on OPG's secretory kinetics.

An association study with haplotype analysis is considered to be a powerful tool for determining a genetic contribution to a common disease. Our analysis of the OPG gene has revealed that four major haplotypes account for 71% of the population 8/16/2007 11:54:40 AMdue to considerable linkage disequilibrium among the six SNPs (Table 6) . With this information, another association analysis could be performed in the future with larger sample size to have a reasonable power to detect an effect. Moreover our finding of the haplotype profile of the gene would be useful information in the study 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 that indicate the relevance of OPG in other conditions, have been reported. First, OPG deficient mice had arterial calcification in the large arteries by 2 weeks of age (Bucay N et al. 1998), indicating a possible role of OPG in disease conditions showing arterial calcification. In addition to that, an association of serum

OPG levels with diabetes and cardiovascular mortality indicates the possibility that OPG may be a cause of vascular calcification (Browner et al. 2001). Second, expression of OPG is not restricted to bone, but 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 et al, 1999). Third, a possible role of OPG in the immune system was shown in experiments with OPG deficient mice (Yun et al. 2001). The authors claimed 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, our OPG Japanese sample studies should be performed with other ethnic groups as OPG might in other populations is also related to osteoporosis.

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Figure Legends

Fig.1 Sequences used for forward and reverse primers are underlined. CA (or GT) repeats are shown in bold.

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Fig.1. Nucleotide sequences of the polymorphic repeats and their flanking region at the OPG gene locus.

a. OPG1

GCACACACGCTCTGTTTCTCTCTCTCTCTGTCTGTCTCTCT
CTCTGTGCATGTGAGTGCTT**TGTGTGTGTGTGTGTGTGTGT**
GTGTGTGTGTGTGTGGAATCAATATAGTAATAAGATATTTA
AAATTGTTAAATCCCAAGTTACCACCCTCC

b. OPG2

AGTCTGGGCAACAGAGCAAGATTTCAT**CACACACACACA**
CACACACACACACACACACACATTAGAAATGTGTACTTGGC
TTTGTTACCTATGGTATTAGTGCATCTATTGCATGGAAC
TTCCAAGCTACTCTGGTTGTGTTAAGCTCTTCATTGGGT
ACAGGTCACTAGTATTAAGTTCAGGTTATTCGGATGCAT
TCCACGGTAGTGATGACAATTCATCAGGCTAG

Table 1. Frequency distribution of CA repeat alleles at 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

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

Status0：concordant unaffected pairs, 1:discordant pairs, 2:concordant affected pairs. Criteria for diagnosis was 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 of the value of allele sharing.

Table 3. Polymorphic status and clinical characteristics

	SNP1			SNP2		
	genotype			genotype		
	TT	TC	CC	GG	GC	CC
n	171	168	55	203	159	32
Age	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

Table 4. Comparison of adjBMD between SNP1 genotype groups

	SNP1 genotype	
	TT	TC+CC
n	171	223
Age	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

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

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:The designation of other SNPs follows the recommendation of mutation nomenclature in the reference (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 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