Sequence Variations in the Osteoprotegerin Gene Promoter in Patients with Postmenopausal Osteoporosis

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Osteoprotegerin (OPG) is a recently discovered member of the TNF receptor superfamily that acts as an important paracrine regulator of bone remodeling. OPG knockout mice develop severe osteoporosis, whereas administration of OPG can prevent ovariectomy-induced bone loss. These findings implicate a role for OPG in the development of osteoporosis.

In the present study, we screened the OPG gene promoter for sequence variations and examined their association with bone mineral density (BMD) in 103 osteoporotic postmenopausal women. Single-strand conformation polymorphism analysis followed by DNA sequencing revealed a presence of four nucleotide substitutions: 209 G→A, 245 T→G, 889 C→T, and 950 T→C.

The frequencies of genotypes were as follows: GG (89.3%), GA (10.7%) for 209 G→A polymorphism; TT (10.7%), TG (10.7%) for 245 T→G polymorphism; and TT (25.2%), TC (33.4%), CC (21.4%) for 950 T→C polymorphism. Substitution 889 C→T was found in only two patients.

Statistically significant association of genotypes with BMD at the lumbar spine (P = 0.005) was observed for 209 G→A and 245 T→G polymorphisms. Haplotype GATG was associated with lower BMD as compared with GTGT haplotype.

Our results suggest that 209 G→A and 245 T→G polymorphisms in the OPG gene promoter may contribute to the genetic regulation of BMD. (J Clin Endocrinol Metab 87: 4080–4084, 2002)

Structural integrity of the skeletal system is maintained by the process of bone remodeling. This complex cellular process involves resorption of old bone by osteoclasts and formation of new bone by osteoblasts, which are tightly coupled under normal conditions. Imbalances between osteoblast and osteoclast activities can be caused by changes in several systemic and local factors, resulting in metabolic bone diseases, such as osteoporosis (1).

Recently, new insight into the regulation of osteoclastogenesis has resulted from the discovery of three members of the TNF and TNF receptor superfamily: receptor activator of nuclear factor-κB ligand (RANKL; Refs. 2 and 3), receptor activator of nuclear factor-κB (RANK; Refs. 4 and 5), and osteoprotegerin (OPG; Refs. 6–10). RANKL activates differentiation of osteoclasts, enhances the activity of mature osteoclasts, and inhibits osteoclast apoptosis by binding to its functional receptor RANK (2, 11, 12). The interaction between RANKL and RANK can be prevented by OPG, which acts as a decoy receptor for RANKL. The biological effects of OPG are opposite to the effects of RANKL and include inhibition of the terminal stages of osteoclastogenesis (6, 7, 9, 10), suppression of mature osteoclast activation (2, 10, 12, 13), and induction of apoptosis (14). So it is actually the ratio of RANKL to OPG that is important for the regulation of osteoclast differentiation and function. It has been proposed that other systemic and local factors could modulate the RANKL/OPG ratio by influencing the expression of RANKL and OPG, and thus regulate osteoclastogenesis and bone resorption (15, 16). Indeed, vitamin D₃, IL-1α, IL-1β, TNF-α and -β, bone morphogenetic protein 2, TGF-β, and 17β-estradiol increase OPG mRNA and protein levels, whereas prostaglandin E₂ and glucocorticoids decrease them (16). The importance of OPG in regulating bone remodeling has also been demonstrated in in vivo models. In OPG knockout mice (17, 18), severe osteoporosis is observed, whereas mice overexpressing OPG (6) develop osteopetrosis and exogenous administration of OPG can prevent ovariectomy-induced bone loss (6).

Because of its important role in the control of bone resorption, the OPG gene is a good candidate gene for osteoporosis. Sequence changes in the promoter region could influence transcription and thus the level of OPG protein. The purpose of the present study was therefore to find out whether there are any sequence variations present in the promoter region of the OPG gene in women with postmenopausal osteoporosis and whether they are associated with bone mineral density (BMD), the most important predictor of osteoporotic fractures.

Patients and Methods

We evaluated 103 consecutive late postmenopausal Slovenian women (in menopause > 6 yr), aged 55–74 yr (mean age, 65.2 yr), who were referred to the outpatient department of Endocrinology clinics for measurement of BMD. Characteristics of the study group are summarized in Table 1. Each patient was examined clinically, and routine biochemical tests were performed to exclude systemic and metabolic bone disease. None had previously taken any drugs known to influence bone metabolism.

The study was approved by the ethical committee, and informed consent was obtained from all of the patients participating in the study.
**DNA analysis**

PCR. DNA was extracted from leukocytes of peripheral blood by the Miller salting-out procedure (19). Oligonucleotide primers were designed based on the sequence of the OPG gene available in GenBank (accession no. AB008821) using NetPrimer 3 software (Fig. 1).

1) Fragment OPG-PR1 (271 bp): sense, 5'-CGA ACC CTA GAG CAA AGT GC-3'; antisense, 5'-TGT CTC ATT GGC CCT AAA GC-3'. Cycling conditions consisted of initial 12 min at 95 C, followed by 35 cycles of 60 sec at 94 C, 30 sec at 56 C, and 60 sec at 72 C, and finally, 7 min at 72 C.

2) Fragment OPG-PR2 (260 bp): sense, 5'-CCC CAG ATA ACA AGG AGT GAA-3'; antisense, 5'-TGT CCC ACA GCT TGG TTC TC-3'. Cycling conditions consisted of initial 12 min at 95 C, followed by 35 cycles of 45 sec at 94 C, 45 sec at 56 C, and 45 sec at 72 C, and finally, 7 min at 72 C.

3) Fragment OPG-PR3 (271 bp): sense, 5'-GTA CGG CCG AAA CTC ACA G-3'; antisense, 5'-GGG AGA GCA GGG GAA AAA-3'. Cycling conditions consisted of initial 5 min at 95 C, followed by 40 cycles of 60 sec at 94 C, 30 sec at 55 C, and 60 sec at 72 C, and finally, 15 min at 72 C.

4) Fragment OPG-PR4 (255 bp): sense, 5'-AGC GCC CTT CCT CCA CGA TTG TCC TC-3'; antisense, 5'-GCT CCG GCA ACA GGA AGT AT-3'. Cycling conditions consisted of initial 12 min at 95 C, followed by 38 cycles of 60 sec at 94 C, 30 sec at 57 C, and 60 sec at 72 C, and finally, 10 min at 72 C.

The PCR mixture (30 µl) contained genomic DNA (100 ng), 1× PCR buffer, 0.2 mM each of the four deoxyribonucleotides, 1.0–2.5 mM MgCl2, 0.42 mM of each of the two oligonucleotide primers, and 0.6 U of AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA) for OPG-PR1, -PR2, and -PR4 fragments or 1.0 U of Taq polymerase (Promega Corp., Madison, WI) for the OPG-PR3 fragment. Aliquots of PCR products were electrophoresed on 2% agarose gel to check their size and quantity.

**Single-strand conformation polymorphism (SSCP) analysis.** PCR products (3 µl) were mixed with 17 LIS loading buffer (10% sucrose, 0.01% bromphenol blue, and 0.01% xylene cyanol), heated at 95–97 C for 3 min, and then transferred immediately to ice. Fifteen microliters were loaded on 8% (37:1) polyacrylamide gels with or without 10% of glycerol. Electrophoresis was run in a Protean II (Bio-Rad Laboratories, Inc., Hercules, CA) electrophoresis unit in 0.5× TBE buffer (50 mM Tris-borate, pH 8.3, and 0.5 mM EDTA) at a constant power of 20 W and at two different temperatures, 9 C or 20 C. After electrophoresis, SSCP patterns were visualized by silver staining.

**DNA sequencing.** PCR products that showed normal and shifted SSCP patterns were purified directly from the PCR using QIAquick PCR purification kit (QIAGEN, Valencia, CA). Their sequences were determined by cycle sequencing using ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Corp.) on an automated DNA-sequencer ABI PRISM 310 (PE Applied Biosystems, Foster City, CA).

**BMD measurement**

BMD at the lumbar spine (L2-L4) and femoral neck were measured by dual-energy x-ray absorptiometry (Lunar Corp., Madison, WI). The precision (coefficient of variation) of this method was 0.5% for the lumbar spine BMD and 1.0% for the femoral neck BMD.

**Statistical analysis**

All data are expressed as mean values ± sd (unless stated otherwise). Data were compared using t test or ANOVA, as appropriate (Statistica 4.1, StatSoft, Inc., Tulsa, OK). P value less than 0.05 was considered statistically significant.

**Results**

One hundred three DNA samples from osteoporotic women were screened for sequence variations in the promoter region of the OPG gene by PCR-SSCP analysis. The 900-bp OPG promoter region, encompassing nucleotides 177 to 1076 (numbered according to the sequence in GenBank accession no. AB008821), was amplified with four primer pairs, creating four overlapping fragments of approximately 260 bp. Differences in SSCP banding patterns were observed in OPG-PR1 and OPG-PR4 fragments, whereas all samples showed a uniform pattern for OPG-PR2 and OPG-PR3 fragments.

To improve detection of sequence variations by SSCP analysis, four different electrophoretic conditions were used. Namely, the sensitivity of SSCP has been reported to range from 30% to over 90% (20) and is affected by various parameters such as gel temperature during electrophoresis, gel matrix composition, gel additives, buffer composition, and size of DNA fragments (21, 22). Changes in any of these parameters can influence the conformation and mobility of single-stranded DNA and thus the probability of distinguishing between wild-type and mutant fragments. In our case, all samples were analyzed at two different temperatures and on gels with or without addition of glycerol (see Patients and Methods). The only condition under which all sequence variations described below could be detected was electrophoresis at 9 C and constant power of 20 W on 8% (37:1) polyacrylamide gels in the absence of glycerol. However, theoretically we cannot exclude the possibility that some variations still remained undetected.

In the OPG-PR1 fragment, two SSCP patterns were observed (Fig. 2). Direct DNA sequencing revealed the presence of two nucleotide variations (Fig. 3). Two other sequence variations described below could be detected in the OPG-PR2 fragment (Fig. 4). In the OPG-PR3 fragment, two sequence variations were observed (Fig. 5). In the OPG-PR4 fragment, no variations were detected (Fig. 6). The DNA sequencing confirmed the presence of single-strand conformation polymorphism patterns for all investigated fragments.

**TABLE 1. Characteristics of the study group (n = 103)**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean (sd)</th>
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<tbody>
<tr>
<td>Age (yr)</td>
<td>65.2 (6.9)</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>157.4 (5.2)</td>
</tr>
<tr>
<td>BMI (kg/m2)</td>
<td>26.8 (3.6)</td>
</tr>
<tr>
<td>Time since menopause (yr)</td>
<td>17.8 (7.5)</td>
</tr>
<tr>
<td>BMD (lumbar spine, g/cm2)</td>
<td>0.717 (0.088)</td>
</tr>
<tr>
<td>T score</td>
<td>-3.323 (0.725)</td>
</tr>
<tr>
<td>BMD (femoral neck, g/cm2)</td>
<td>0.733 (0.110)</td>
</tr>
<tr>
<td>T score</td>
<td>-1.897 (0.967)</td>
</tr>
</tbody>
</table>
of two sequence variations, 209 G→A and 245 T→G. The frequencies of genotypes were as follows: GG (89.3%), GA (10.7%) for 209 G→A polymorphism; and TT (89.3%), TG (10.7%) for 245 T→G polymorphism. Because all samples tested were either homozygotic with nonmutated GGTT haplotype or heterozygotic with GATG haplotype, only two groups of patients for statistical evaluation of both polymorphisms were available. The two groups (GGTT and GATG) did not differ in chronological age (65.0 ± 7.2 vs. 66.9 ± 3.9 y; P = 0.44), menopausal age (17.6 ± 7.7 vs. 19.8 ± 4.7 y; P = 0.44), or body mass index (BMI) (26.9 ± 3.7 vs. 26.8 ± 2.5 kg/m²; P = 0.95). They did not differ in BMD of the femoral neck region, but had significantly different BMD in the lumbar region (Fig. 3A).

In the OPG-PR4 fragment, four SSCP patterns were observed (Fig. 4). Direct DNA sequencing revealed the presence of two sequence variations, 889 C→T and 950 T→C. Substitution 889 C→T was found in only two patients. The frequencies of genotypes for 950 T→C polymorphism were as follows: TT (25.2%), TC (53.4%), and CC (21.4%). Stratifying our patients according to 950 T→C polymorphism, three groups of patients emerged (TT, TC, and CC). Again, there were no differences in chronological age (64.7 ± 6.8 vs. 66.1 ± 6.9 vs. 63.5 ± 7.0 y; P = 0.24), menopausal age (17.8 ± 8.5 vs. 18.5 ± 6.8 vs. 15.4 ± 7.5 y; P = 0.45), or BMI (27.5 ± 2.9 vs. 26.9 ± 3.7 vs. 25.3 ± 3.0 kg/m²; P = 0.19). Moreover, differences in BMD were not statistically significant (Fig. 3B).

**Discussion**

PCR-SSCP screening of the OPG gene promoter in patients with osteoporosis indicated presence of several sequence variations. With direct DNA sequencing, the following nucleotide substitutions were identified, 209 G→A, 245 T→G, 889 C→T, and 950 T→C. For two of them, 209 G→A and 245 T→G, correlation with BMD could be demonstrated. Patients with GGTT haplotype had significantly greater BMD in lumbar region than patients with GATG haplotype.

So far, only a few reports in the form of conference abstracts are available, in which the association of OPG gene promoter polymorphisms with bone metabolism has been studied (23–26). The 209 G→A polymorphism has not been described before. Here, only nonmutated homozygotes (GG, 89.3%) and heterozygotes (GA, 10.7%) were observed. Another polymorphism characterized by a nucleotide substitution 245 T→G was identified only 36 nucleotides downstream. Here again, only nonmutated homozygotes and heterozygotes were found. Interestingly, the distribution of genotypes was exactly the same as for the 209 G→A polymorphism. Thus, only two haplotypes were observed, TTGG and TGGA. This was confirmed by restriction fragment length polymorphism analysis of all samples with TaqI and Hinfl restriction endonuclease (results not shown), because 209 G→A introduces a Hinfl restriction site, and 245

![Fig. 2. SSCP analysis of OPG-PR1 fragment of the OPG gene promoter. Samples with migration pattern A are nonmutated homozygotes, whereas samples with migration pattern B are heterozygous for both 209 A→G and 245 T→G nucleotide substitutions. ss, Single-stranded DNA.](image1)

![Fig. 3. Correlation of the OPG gene promoter polymorphisms with BMD. A, BMD at the lumbar spine and femoral neck in the two groups of patients stratified according to 209 G→A and 245 T→G polymorphisms. B, BMD at the lumbar spine and femoral neck in the three groups of patients stratified according to 950 T→C polymorphism. Values of BMD are expressed as mean ± SD.](image2)

![Fig. 4. SSCP analysis of OPG-PR4 fragment of the OPG gene promoter. Samples with migration pattern A are nonmutated homozygotes, samples with migration pattern B are heterozygotes, and samples with migration pattern C are mutated homozygotes for 950 T→C nucleotide substitution. Migration pattern D corresponds to 950 TC heterozygote and mutated homozygote for 889 T→C nucleotide substitution. ss, Single-stranded DNA.](image3)
T→G a TaqI restriction site. The 245 T→G polymorphism has already been described by Langdahl et al. (23). They reported a higher frequency of mutant G allele in osteoporotic patients than in healthy controls, which could suggest a possible association of this polymorphism with BMD. This is in accordance with our results, because lower BMD values at lumbar spine were found in patients with the GATG haplotype. However, there was no association with femoral neck BMD. This could be explained by the fact that trabecular bone, which is mainly present in vertebral bone, is more metabolically active and reflects imbalances in bone remodeling earlier than cortical bone, which predominates in femoral neck.

The 889 C→T substitution has also not been described before, but it was quite uncommon, because only two patients were TT homozygotes and all the rest were CC homozygotes. Therefore it was not included in the statistical analyses.

Finally, our results confirmed the presence of the 950 T→C polymorphism that has already been described as the HincII polymorphism (23–26). The frequencies of genotypes were: TT (25.2%), TC (53.4%), and CC (21.4%). Specific SSCP bands could be assigned to each allele, so it was not necessary to perform HincII restriction fragment length polymorphism analysis of all samples (three examples of each genotype were tested to confirm the results obtained by SSCP). In contrast to 209 G→A and 245 T→G polymorphism, no association of 950 T→C polymorphism with BMD at either lumbar spine or femoral neck could be observed. Our results are consistent with scarce data in literature (24–26). In contrast to these findings, only Langdahl et al. (23) found that CC genotype of 950 T→C polymorphism was associated with higher bone mass than TT genotype.

Sequence variations in the promoter region of the OPG gene could result in altered binding of different transcription factors and could thus affect the expression of OPG. Any change in OPG concentration in the bone microenvironment may influence the RANKL/OPG ratio and disturb the balanced process of bone remodeling. The OPG promoter contains several putative transcription factor binding sites, and the functions of few of them have been demonstrated. Thirunavukkarasu et al. (27) identified two regions in the OPG promoter, the Cbfα1-binding element (OSE3) and the Smad binding element, which are responsible for mediating TGF-β effects. Similarly, Wang et al. (28) identified two Hoxc-8 binding sites that mediate OPG promoter activity in response to stimulation with bone morphogenetic proteins. The polymorphisms discovered in our study do not lie in any of the functional transcription factor binding sites that could lead to similar conclusions as to their role in regulating OPG expression. Demonstration of their possible function would require in vitro experiments on cell cultures.

In this preliminary study, the main purpose was to identify sequence variations in the OPG promoter. In addition to demonstration of two new variants, 209 G→A and 889 C→T, our data suggest that polymorphisms of the OPG promoter are one of the genetic determinants of bone mass, and haplotype 209GA/245TG could be considered as a risk factor for genetic susceptibility to postmenopausal osteoporosis. The main limitation of our study is the relatively small number of samples tested, which diminishes its statistical power and the possibility of detecting correlations, especially in such a complex disease as osteoporosis. Further studies on larger numbers of samples, possibly from pre-, peri-, and postmenopausal women, are necessary to follow up these interesting suggestions. The association with other factors such as markers of bone metabolism, bone quality, and also other candidate genes should also be tested.

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