Osteoporosis is defined as a skeletal disorder characterized by compromised bone strength predisposing a person to an increased risk of fracture (1). It has been estimated that bone mineral density (BMD) accounts for approximately 70% of bone strength, and that at least 70% of BMD variance is attributed to genetic factors (2). A number of candidate genes have been investigated in terms of their potential involvements in the regulation of bone mass in various populations, but the results have been conflicting (3, 4) and osteoporosis genes have not been defined yet. To date, we have demonstrated that the estrogen receptor (ER) PvuII and XbaI polymorphisms (5), the insulin-like growth factor (IGF-I) (CA) polymorphism (6), and the vitamin D receptor (VDR) baTL haplotype (7) and the osteoprotegerin (OPG) gene G1181C polymorphism (8), are candidate genetic factors in Korean women.

It has been suggested that bone mass may be affected by multiple genes, and therefore, further studies on other candidate genes are required.

The Wnt/β-catenin signaling pathway has been shown to play a major role in the control of bone formation, and is specifically involved in osteoblast proliferation, differentiation, and apoptosis (9-12). In order to transduce the canonical signal, Wnts bind to two different receptors: the low density lipoprotein receptor-related protein (LRP) 5/6 and frizzled receptor (FZD) family members. Recently it has been reported that single nucleotide polymorphisms (SNPs) in the LRP gene may be associated with bone mass, depending on ethnicity (13, 14). We have recently reported non-association of LRP SNPs with bone mass in postmenopausal Korean women (15).

There are 10 members of the FZD gene family in humans. It has been suggested that not all FZD have the same functions in the Wnt signaling pathway, indicating the existence of inhibitory and stimulatory FZD (9). Furthermore it has been reported that the Wnt pathway might be associated with OPG-receptor activator of the NF-κB (RANK) system which is known to regulate osteoclast formation and activity (16, 17). The aim of the present study was to investigate the association between polymorphisms of the FZD genes, and circulating OPG, soluble RANK ligand (sRANKL) levels, bone turnover marker levels, and BMD in postmenopausal Korean women.

We investigated the association between single nucleotide polymorphisms (SNPs) in the frizzled (FZD) genes in the Wnt signal pathway and circulating osteoprotegerin (OPG), soluble receptor activator of NF-κB ligand (sRANKL) levels, bone turnover markers, and bone mineral density (BMD) in postmenopausal women. The SNPs in the FZD1, FZD5, FZD6, FZD7, and FZD9 genes were analyzed by direct sequencing in 371 postmenopausal Korean women. Levels of serum OPG, sRANKL, osteocalcin, C-telopeptide of type I collagen, calcium, parathyroid hormone and calcitonin, and BMD at the lumbar spine and femoral neck were measured. The SNPs in the FZD1, FZD5, FZD7, and FZD9 genes, and in exon 2 of the FZD6 gene were not observed. No significant differences in the adjusted BMD of lumbar spine and femoral neck and serum levels of OPG, sRANKL, and bone markers were noted among the single or haplotype genotypes of the L345M and E664A SNPs in the FZD6 gene and the distributions of these single or haplotype genotypes were not different according to the bone mass status. In conclusion, the polymorphisms of the FZD genes are not associated with BMD of the lumbar spine and femoral bone, bone turnover markers, or circulating OPG-sRANKL in Korean women.

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Key Words: Bone Density; Frizzled Receptors; Polymorphism, Genetic; Osteoprotegerin; Biological Markers

INTRODUCTION

Osteoporosis is defined as a skeletal disorder characterized by compromised bone strength predisposing a person to an increased risk of fracture (1). It has been estimated that bone mineral density (BMD) accounts for approximately 70% of bone strength, and that at least 70% of BMD variance is attributed to genetic factors (2). A number of candidate genes have been investigated in terms of their potential involvements in the regulation of bone mass in various populations, but the results have been conflicting (3, 4) and osteoporosis genes have not been defined yet. To date, we have demonstrated that the estrogen receptor (ER) PvuII and XbaI polymorphisms (5), the insulin-like growth factor (IGF-I) (CA) polymorphism (6), and the vitamin D receptor (VDR) baTL haplotype (7) and the osteoprotegerin (OPG) gene G1181C polymorphism (8), are candidate genetic factors in Korean women. It has been suggested that bone mass may be affected by multiple genes, and therefore, further studies on other candidate genes are required.

The Wnt/β-catenin signaling pathway has been shown to play a major role in the control of bone formation, and is specifically involved in osteoblast proliferation, differentiation, and apoptosis (9-12). In order to transduce the canonical signal, Wnts bind to two different receptors: the low density lipoprotein receptor-related protein (LRP) 5/6 and frizzled receptor (FZD) family members. Recently it has been reported that single nucleotide polymorphisms (SNPs) in the LRP gene may be associated with bone mass, depending on ethnicity (13, 14). We have recently reported non-association of LRP SNPs with bone mass in postmenopausal Korean women (15).

There are 10 members of the FZD gene family in humans. It has been suggested that not all FZD have the same functions in the Wnt signaling pathway, indicating the existence of inhibitory and stimulatory FZD (9). Furthermore it has been reported that the Wnt pathway might be associated with OPG-receptor activator of the NF-κB (RANK) system which is known to regulate osteoclast formation and activity (16, 17). The aim of the present study was to investigate the association between polymorphisms of the FZD genes, and circulating OPG, soluble RANK ligand (sRANKL) levels, bone turnover marker levels, and BMD in postmenopausal Korean women.
**MATERIALS AND METHODS**

**Subjects**

The current study included 371 postmenopausal women between the ages of 48 and 70 yr, who attended the Menopause Clinic of Seoul National University Hospital for bone mass examination and who agreed to participate. All of the subjects had been without spontaneous menses for at least one year, and underwent a careful physical examination, and a medical history review. Blood glucose levels, and hepatic and renal functions were determined in the participants. Women who had undergone bilateral oophorectomy or women with current hepatic disease, renal disease, or diabetes mellitus were excluded. None of the subjects had received any medication known to affect bone metabolism before the study. All subjects provided their informed consent, and the study protocol was approved by the Institutional Review Board of Seoul National University Hospital.

**Measurements of BMD and body mass index (BMI)**

The BMD was measured in grams per square centimeter at the lumbar spine (L2-L4), femoral neck, trochanter, and Ward’s triangle, using a Lunar DPX-L dual-energy radiography absorptiometer (Lunar Radiation Corp., Madison, WI, U.S.A.), and categorized into the following three groups according to the criteria of the World Health Organization (18) as, normal, osteopenic, or osteoporotic, relative to the means and standard deviation of young adult Korean women. The in vivo coefficient of variation was 1.4% for the lumbar spine, 2.1% for the femoral neck, 1.1% for the trochanter, and 2.1% for Ward’s triangle. The BMI was calculated by dividing body weight (kg) by the square of body height (m²).

**Measurements of bone turnover markers**

Blood samples were collected from all of the subjects in accordance with the guidelines of the Declaration of Helsinki. Serum osteocalcin (OST) was measured using a competitive radioimmunoassay kit (Techno Genetics, Milano, Italy). The minimum detection limit was 0.6 ng/mL, and intra- and inter-assay variations for OST were 4.0% and 5.1%, respectively. Serum carboxy-terminal C-telopeptide of type I collagen (CTX) was measured using a serum CrossLaps One Step enzyme-linked immunosorbent assay kit (Osteometer Biotech, Herlev, Denmark). The minimum detection limit was 94 pM/L and intra- and inter-assay variations for CTX were 5.4% and 5.1% respectively.

**Measurement of serum calcitonin, parathyroid hormone (PTH), and calcium (Ca)**

Calcitonin was measured using an immunoradiometric assay kit (CIS bio international, Yvette Cedex, France). The minimum detection limit was 1.5 pg/mL, and intra- and inter-assay variations for calcitonin were 3.4% and 3.1%, respectively. PTH was measured using a total intact PTH immunoradiometric assay kit (IBL, Hamburg, Germany). The minimum detection limit was 1 pg/mL and intra- and inter-assay variations for PTH were 2.7% and 3.4%, respectively. Ca was measured by an atomic extinction photometer.

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**Table 1. FZD family SNPs studied and their PCR primers**

| Types of FZD | dbsNP ID | Locations | Base changes | Amino acid changes | PCR primers |
|--------------|----------|-----------|--------------|--------------------|-------------|
| FZD1         | rs3752146| Exon 1    | A/G          | Met 343 Val        | F: GTGAGCCGACCAAGGTGTAT  
R: RCTATAGAAGGATGTCGAG |
| FZD5         | rs1056614| Exon 2    | A/T          | Thr 88 Ser         | F: CAAACCAGTCAACCCAGACA  
R: CGGGTGAATTCATGCAGAG |
| FZD6         | rs827528  | Exon 2    | G/A          | Val 33 Met         | F: TTGATGGGATCTTCTAGAG  
R: GCGCAATACCTGCGCATAA |
| rs3806553    | Exon 4    | C/A       | Leu 345 Met  |                     | F: ATAGGCTTGCACCGTTT   
R: AAAAGCACAGTGCCAAAGAT |
| rs12549394   | Exon 7    | A/C       | Glu 664 Ala  |                     | F: TATGACACTCCGACTAGCA  
R: AACCACTTCCTGCTTCTTT |
| FZD7         | rs1154613 | Exon 1    | C/T          | Leu 282 Phe        | F: CCCACTGTCCTACCTACCC  
R: ATGAATGAGCCAGCCGACAG |
| FZD9         | rs17852398| Exon 1    | G/C          | Arg 199 Pro        | F: CCCATGCTGGACGAGTCA   
R: GAAGGCCGTGAGGAAAGAG |
| rs17856756   | Exon 1    | G/A       | Val 448 Ile  |                     | F: CTGGTGGCAGTGGAGTTTT  
R: GCTAGAGGTTGTGTTGGGGTTC |
| rs17856757   | Exon 1    | A/G       | Asn 524 Ser  |                     | F: same as F1           
R: same as R1 |
| rs17852397   | Exon 1    | A/G       | His 556 Arg  |                     | F: same as F1           
R: same as R1 |

FZD, frizzled; SNPs, single nucleotide polymorphisms; PCR, polymerase chain reaction.
Measurement of serum OPG and sRANKL

Serum OPG was measured using an enzyme-linked immunosorbent assay kit (R&D, Minneapolis, MN, U.S.A.). The minimum detection limit was 0.06 ng/mL and inter- and intra-assay variations for OPG were 7.0% and 3.9%, respectively. Serum sRANKL was measured using an enzyme immunoassay kit (Biomedica Gruppe, Wien, Austria). The minimum detection limit was 1.6 pg/mL and the inter- and intra-assay variation for sRANKL were 7.2% and 3.9%, respectively.

Determination of the polymorphisms of the FZD genes

Genomic DNA was extracted from the peripheral blood leukocytes using a QIAamp DNA blood kit (Qiagen GmbH, Hilden, Germany). The polymorphic regions of the FZD genes were amplified by polymerase chain reaction (PCR) with specific forward and reverse primers as shown in Table 1. PCR products were electrophoresed through 1.5% agarose gel to verify the reaction, and purified directly from PCR reaction products were electrophoresed through 1.5% agarose gel to verify the reaction, and purified directly from PCR reaction using a PCR clean kit (Qiagen GmbH, Hilden, Germany). The polymorphic regions of the FZD genes were amplified by polymerase chain reaction (PCR) with specific forward and reverse primers as shown in Table 1. PCR products were electrophoresed through 1.5% agarose gel to verify the reaction, and purified directly from PCR reaction using a PCR clean kit (Qiagen GmbH, Hilden, Germany). The polymorphic regions of the FZD genes were amplified by polymerase chain reaction (PCR) with specific forward and reverse primers as shown in Table 1. PCR products were electrophoresed through 1.5% agarose gel to verify the reaction, and purified directly from PCR reaction using a PCR clean kit (Qiagen GmbH, Hilden, Germany). The polymorphic regions of the FZD genes were amplified by polymerase chain reaction (PCR) with specific forward and reverse primers as shown in Table 1. PCR products were electrophoresed through 1.5% agarose gel to verify the reaction, and purified directly from PCR reaction using a PCR clean kit (Qiagen GmbH, Hilden, Germany). The polymorphic regions of the FZD genes were amplified by polymerase chain reaction (PCR) with specific forward and reverse primers as shown in Table 1. PCR products were electrophoresed through 1.5% agarose gel to verify the reaction, and purified directly from PCR reaction using a PCR clean kit (Qiagen GmbH, Hilden, Germany).

Statistical analyses

All data are expressed as mean $\pm$ SE. All statistical analyses were performed using the Statistical Package for Social Science (SPSS, Chicago, IL, U.S.A.). Genotype frequencies for each polymorphism were tested against the Hardy-Weinberg equilibrium. Linkage disequilibria resulting from the non-random associations of genotypes at polymorphic sites, were also assessed by the chi-square test. Differences in anthropometric characteristics between the different FZD genotypes or haplotypes were tested using the Student’s t-test or one way analysis of variance (ANOVA), followed by Tukey’s multiple comparison test. Similar comparisons were made after adjusting the BMD and the levels of OPG, sRANKL or biochemical markers for potential confounding factors such as age, years since menopause, and BMI, using analysis of covariance (ANCOVA). A P value of less than 0.05 was considered to be significant for all analyses.

RESULTS

Single polymorphisms

The SNPs in the FZD1, FZD5, FZD7, and FZD9 genes, and in exon 2 of the FZD6 gene were not observed. The distributions of the L345M SNP in the exon 4 and the E664A SNP in the exon 8 of FZD6 gene were as follows: LL 36.4%, LM 43.7%, MM 19.9%, AA 95.7%, and AE 4.3%. These allele and genotype frequencies did not deviate from the Hardy-Weinberg equilibrium.

After adjusting for potential confounding factors, such as age, BMI, and years since menopause, no significant differences in the BMD of the lumbar spine and proximal femur were noted among the genotypes of the L345M and the E664A SNPs in FZD6 gene (Table 2). As shown in Table 3, the prevalences of genotypes of these FZD6 polymorphisms were not different among normal, osteopenic and osteoporotic postmenopausal women.

No statistically significant differences in the adjusted serum levels of OPG, or sRANKL, or sRANKL $\times$ 1,000/OPG ratios were found for these genotypes of the FZD6 gene polymorphisms (Table 4). Similar findings were obtained for the adjusted serum levels of bone turnover markers, such as, CTX, OST, PTH, calcitonin, and Ca.

Table 2. Clinical characteristics and bone mineral density in postmenopausal women in relation to the FZD6 SNPs

| Exon 4 L345M | Exon 7 E664A |
|-------------|-------------|
| LL (n=135)  | LM (n=162)  | MM (n=74) | P  | AA (n=355)  | AE (n=16) | P*       |
| Age (yr)    | 58.0 $\pm$ 0.6 | 57.7 $\pm$ 0.5 | 57.2 $\pm$ 0.7 | 0.72 | 57.8 $\pm$ 0.3 | 57.6 $\pm$ 1.5 | 0.90 |
| Years since menopause | 9.4 $\pm$ 0.6 | 9.1 $\pm$ 0.6 | 9.1 $\pm$ 0.7 | 0.94 | 9.2 $\pm$ 0.4 | 9.0 $\pm$ 1.3 | 0.90 |
| Weight (kg) | 58.7 $\pm$ 0.7 | 57.9 $\pm$ 0.5 | 58.1 $\pm$ 0.7 | 0.66 | 58.4 $\pm$ 0.4 | 57.0 $\pm$ 1.3 | 0.44 |
| Height (cm) | 155.2 $\pm$ 0.4 | 155.3 $\pm$ 0.4 | 155.8 $\pm$ 0.6 | 0.63 | 155.4 $\pm$ 0.2 | 153.9 $\pm$ 1.0 | 0.22 |
| BMI (kg/m$^2$) | 24.4 $\pm$ 0.3 | 24.0 $\pm$ 0.2 | 24.0 $\pm$ 0.3 | 0.49 | 24.1 $\pm$ 0.2 | 24.1 $\pm$ 0.6 | 0.92 |
| Bone mineral density (g/cm$^2$) | 1.002 $\pm$ 0.015 | 1.009 $\pm$ 0.015 | 0.999 $\pm$ 0.020 | 0.92 | 1.005 $\pm$ 0.009 | 0.998 $\pm$ 0.046 | 0.89 |
| Lumbar spine | 0.813 $\pm$ 0.013 | 0.817 $\pm$ 0.010 | 0.830 $\pm$ 0.017 | 0.69 | 0.816 $\pm$ 0.007 | 0.819 $\pm$ 0.033 | 0.97 |
| Femoral neck | 0.711 $\pm$ 0.010 | 0.732 $\pm$ 0.014 | 0.741 $\pm$ 0.019 | 0.87 | 0.725 $\pm$ 0.008 | 0.757 $\pm$ 0.037 | 0.42 |
| Ward’s triangle | 0.631 $\pm$ 0.013 | 0.660 $\pm$ 0.011 | 0.649 $\pm$ 0.020 | 0.26 | 0.647 $\pm$ 0.008 | 0.674 $\pm$ 0.046 | 0.47 |

P, ANCOVA; *P*, Student’s t-test; $^*$Values, adjusted for age, years since menopause, and BMI. Values are mean $\pm$ SE.

FZD, frizzled; SNPs, single nucleotide polymorphisms; BMI, body mass index.
Combined polymorphisms

Strong linkage disequilibrium was found between the L345M and E664A polymorphisms ($\chi^2=9.032$, $P<0.05$). Three different haplotype alleles (ME, MA, and LA) were identified by haplotype analysis for these two polymorphisms of the FZD 6 gene in combination, and these haplotypes combined to give five different genotypes: LAMA (42.0%), LALA (35.8%), MAMA (18.1%), MELA (2.2%), and MAME (1.9%). However, these haplotype alleles or genotypes were found to have no significant association with the adjusted BMDs of all skeletal sites measured, or with the serum levels of OPG, RANKL, CTX, OST, PTH, calcitonin, and Ca.

### DISCUSSION

It has been demonstrated that the canonical Wnt/β-catenin pathway modulates most aspects of osteoblast physiology, including proliferation, differentiation, bone matrix formation and mineralization, and apoptosis. This signaling is initiated following binding of Wnt proteins to the FZD and LRP5/6 receptors (9-12). To the best of our knowledge, this is the first study conducted to establish an association between bone mass and the SNPs in the FZD genes.

We investigated non-synonymous SNPs in the FZD1, FZD5, FZD6, FZD7, and FZD9 genes, which have been registered in the SNP database by the National Center For Biotechnology Information (dbSNP). The SNPs in the FZD1, FZD5, FZD7, and FZD9 genes, and in exon 2 of the FZD6 gene were not polymorphic in our study subjects. The frequencies of the L allele of the L345 SNP and the E allele of the E664A SNP in the FZD6 gene were comparable to that reported in Asian dbSNP.

FZD6 is located on chromosome 8q22.3 (19). It has been reported that the FZD6 transcript is detected in calvaria and primary osteoblast (20), and decreased in osteoblasts after 10 days of culture in conditions favoring mineralization (9). Human FZD6 acts as a negative regulator of the canonical Wnt/β-catenin signaling cascade (21), which is believed to play a substantial role in the control of bone formation. In the present study, no significant association between C345A

### Table 3. Distribution of single genotype of the FZD6 SNPs according to the bone mass status

| Polymorphic sites | Genotypes | Normal (n=93) | Osteopenia (n=175) | Osteoporosis (n=103) | Cross-tabulation test |
|-------------------|-----------|---------------|-------------------|---------------------|----------------------|
| Exon 4 L345M      | LL        | 30 (32.3%)    | 68 (38.9%)        | 36 (35.0%)          | $P=0.54$             |
|                   | LM        | 46 (49.5%)    | 71 (40.6%)        | 44 (44.7%)          | $P=0.37$             |
|                   | MM        | 17 (18.3%)    | 36 (20.6%)        | 21 (20.4%)          | $P=0.90$             |
| Exon 7 E664A      | AA        | 90 (96.8%)    | 167 (95.4%)       | 98 (95.1%)          |                      |
|                   | AE        | 31 (3.2%)     | 8 (4.6%)          | 5 (4.9%)            |                      |

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FZD, frizzled; SNPs, single nucleotide polymorphisms.

### Table 4. Serum levels of OPG, RANKL and bone turnover markers in postmenopausal women in relation to FZD6 SNPs

| Polymorphic sites | Genotypes | Normal (n=93) | Osteopenia (n=175) | Osteoporosis (n=103) | Cross-tabulation test |
|-------------------|-----------|---------------|-------------------|---------------------|----------------------|
| Exon 4 L345M      | LL        | 9,336.5±277.4 | 9,083.4±319.8     | 9,734.4±489.4       | 0.47                 |
|                   | LM        | 7.4±0.7       | 7.7±0.7           | 10.6±3.3            | 0.30                 |
|                   | MM        | 0.8±0.1       | 1.0±0.1           | 1.3±0.5             | 0.36                 |
| Exon 7 E664A      | AA        | 9,317.9±191.4 | 9,143.6±1,112.2   | 1.0±0.1             | 0.61                 |
|                   | AE        | 8.3±0.8       | 5.6±0.2           | 8.3±0.8             | 0.46                 |

Serum bone markers

| Serum OPG-sRANKL | LL (n=90) | LM (n=105) | MM (n=41) | $P$ |
|------------------|-----------|-----------|-----------|-----|
| OPG (pg/mL)      | 1,438.1±152.3 | 2,412.6±651.0 | 1,296.9±299.4 | 0.24 |
| sRANKL (pg/mL)   | 13.6±1.0  | 15.0±0.6  | 13.6±1.2  | 0.25 |
| sRANKL×1,000/OPG | 10.6±1.0  | 9.9±0.7  | 9.3±1.3  | 0.63 |

Calcitonin (pg/mL)

| Serum bone markers | LL (n=90) | LM (n=105) | MM (n=41) | $P$ |
|--------------------|-----------|-----------|-----------|-----|
| CTX (pM/L)         | 13.6±1.0  | 15.0±0.6  | 13.6±1.2  | 0.25 |
| OST (ng/mL)        | 9.9±0.7  | 9.3±1.3  | 9.3±1.0  | 0.07 |
| PTH (pg/mL)        | 48.0±0.4 | 7.0±1.8  | 7.0±1.8  | 0.24 |
| Ca (mg/dL)         | 5.4±0.2 | 9.4±0.2  | 9.4±0.2  | 0.23 |

$P$, ANCOVA; $P^*$, Student’s t-test.

Values are mean±SE.

OPG, osteoprotegerin; RANKL, receptor activator of the NF-κB (RANK) ligand; FZD, frizzled; SNPs, single nucleotide polymorphisms; CTX, CrossLaps; OST, osteocalcin; PTH, parathyroid hormone; Ca, calcium.

Combined polymorphisms

Strong linkage disequilibrium was found between the L345M and E664A polymorphisms ($\chi^2=9.032$, $P<0.05$). Three different haplotype alleles (ME, MA, and LA) were identified by haplotype analysis for these two polymorphisms of the FZD 6 gene in combination, and these haplotypes combined to give five different genotypes: LAMA (42.0%), LALA (49.5%), MAMA (18.1%), MELA (2.2%), and MAME (1.9%). However, these haplotype alleles or genotypes were found to have no significant association with the adjusted BMDs of all skeletal sites measured, or with the serum levels of OPG, RANKL, CTX, OST, PTH, calcitonin, and Ca.
or A664C SNPs in the FZD6 gene and BMD at skeletal sites measured or circulating levels of bone turnover markers were noted. Haplotype analysis on the L345M and E664A SNPs in combination showed similar findings. Our results may indicate that SNPs in FZD6 gene may not be involved in the regulation of bone mass or bone remodeling.

It has been demonstrated that the secreted antagonist of the Wnt/β-catenin signaling pathway regulates the expression of OPG- RANKL of bone tissue (16). Secreted FZD related protein, which is highly homologous to the Wnt binding protein of FZD and is capable of binding Wnt, has been shown to bind RANKL, resulting in inhibition of osteoclastogenesis (16). In this study, SNPs in FZD6 gene did not affect circulating OPG-sRANKL levels.

The present study had several limitations. First, this study group was composed of ethnically homogenous Korean women and undertaken in relatively small number of women who attended a menopause clinic. Second, limited numbers of SNP in the FZD genes were analyzed. Therefore, further studies including other SNPs in the FZD genes are necessary in a larger general population and in other ethnic groups.

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