The relationship between Receptor Activator of Nuclear Factor-κB Ligand (RANKL) gene polymorphism and aortic calcification in Korean women

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Abstract. The aim of this study was to investigate the relationship between RANKL gene polymorphisms and aortic calcification in Korean women. In 237 healthy Korean women, aortic calcification in thoracic and abdominal aorta was examined in simple radiologic method and lumbar spine and femoral neck BMD were examined by dual energy X-ray absorptiometry. Serum OPG levels, bone turnover markers, such as ALP levels and urine deoxypyridinoline levels, and urine calcium excretion were measured. Genotyping of two RANKL gene polymorphisms, rs2277438 and rs9594782, was performed by allelic discrimination using the 5' nuclease polymerase chain reaction assay. The subjects with CT/CC genotypes of the rs9594782 polymorphism had a 3.9 times higher risk of aortic calcification compared with TT genotype. This significance was significant even after adjustment for age, BMI, blood pressure, fasting plasma glucose, serum high- and low-density lipoprotein cholesterol levels. Mean levels of urine deoxypyridinoline were significantly higher in the subjects with AG/GG genotypes of the rs2277438 polymorphism compared with AA genotype, and this significance was persistent even after adjustment for age and BMI. There were no associations of mean values for age, BMI, serum OPG and ALP levels, urine calcium excretion, and BMD with RANKL gene polymorphisms. The RANKL gene rs9594782 polymorphism was associated with aortic calcification in Korean women. Rs2277438 polymorphism showed significant association with urine deoxypyridinoline levels, a bone resorption marker. These results suggest its role on vascular calcification and bone metabolism in humans.

Key words: Receptor activator of nuclear factor-κB ligand (RANKL), Polymorphism, Aortic calcification

AORTIC CALCIFICATION was associated with aging, hypertension, diabetes, hypercholesterolemia, and osteoporosis, leading to serious clinical consequences, such as coronary artery disease, stroke, peripheral arterial disease, and cardiovascular mortality [1-6]. One possible mechanism emerging in vascular calcification is the receptor activator of NF-κB ligand (RANKL)/osteoprotegerin (OPG) system, molecules of the tumor necrosis factor (TNF)-related family recently discovered to be key regulators of osteoclastogenesis [7, 8].

RANKL, a 316-amino acid transmembrane protein, is highly expressed by osteoblasts in bone and T-cells in lymphoid tissue, and also exist in circulation in biologically active soluble forms [9,10]. RANKL binds to receptor activator of NF-κB (RANK), a 616-amino acid transmembrane receptor, on the surface of osteoclasts, and their interaction activates osteoclast differentiation, enhances the activity of mature osteoclasts, and inhibits osteoclasts apoptosis [9-12]. OPG, a 380-amino acid soluble receptor widely expressed by osteoblasts and vascular cells, acts as a decoy receptor...
for RANKL and its biological effects are opposite to the effects of RANKL [13]. RANKL-knockout mice show severe osteopetrosis, no tooth eruption, markedly reduced skeletal growth and completely lack osteoclastogenesis due to the inability of osteoblasts to support osteoclastogenesis [14]. OPG-knockout mice also show severe osteoporosis with extensive calcification in the aorta and renal artery [13, 15]. These phenotypes address the significant role of RANKL/OPG system in bone metabolism and vascular calcification.

Both levels of serum RANKL and OPG have been reported to be significantly associated with the cardiovascular disease and mortality [16, 17], nontraumatic fracture [18] and osteoporosis [19]. Protection against mineralization in the vessel wall is thought to be achieved by a modulation of OPG and RANKL expression, causing inhibition of osteoclast maturation, thus preventing subsequent release of calcium and mineral from bone [20]. Recently published study reports the role of RANKL in the acceleration of vascular smooth muscle cell mineralization, which is inhibited by the addition of OPG [21].

OPG gene polymorphisms with increased risk for coronary artery disease and osteoporotic fracture were reported [22-25], and recently, RANKL gene polymorphisms have been studied relative to bone mineral density and osteoporotic fracture [24, 26, 27]. However, there has been no reported data on the association of cardiovascular system with RANKL gene polymorphisms. Here, we genotyped two single nucleotide polymorphisms (SNPs) in the RANKL gene, rs2277438 and rs9594782, which have been studied in relation with bone metabolism in apparently healthy Korean women [26, 27], and investigated their association with aortic calcifications in thoracic and abdominal aorta, to see if RANKL gene polymorphisms are associated with vascular calcification.

Methods

Study subjects

Among the subjects who underwent health check-ups from January 1, 2002 to December 31, 2002 at Miz Medi Hospital, Seoul, Korea, 237 female subjects (mean age 51.3±6.9 years) were selected. Subjects with diseases affecting cardiovascular system including coronary heart diseases, cerebrovascular diseases, thyroid diseases, parathyroid diseases, pituitary diseases, adrenal diseases, diabetes, chronic renal and hepatic diseases, alcohol abuse and malignancy, and those taking medications such as statins, corticosteroid, thyroid hormones and estrogen, all of which are known to affect cardiovascular system, were excluded. The subjects included 123 premenopausal women and 114 postmenopausal women. Menopause was defined as the absence of menstruation for 12 months after the last menstruation and a follicle stimulating hormone (FSH) level higher than 40 mIU/mL.

Written informed consent was obtained from each participant and the study protocol confirms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the Institutional Review Board of Miz Medi Hospital.

Anthropometric measurement, blood chemistry and the measurement of serum OPG concentration

We measured height, weight, and systolic and diastolic blood pressures, and body mass index (BMI) was calculated as weight (kg) divided by height (m) squared and was used as an index of obesity (kg/m²).

After 12 hours of fasting, fasting plasma glucose, serum total cholesterol, triglyceride, high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) levels were checked. The hexokinase method was used to measure glucose levels and an enzymatic calorimetric test was used to measure total cholesterol and triglyceride levels. The selective inhibition method was used to measure the level of HDL-C and a homogeneous enzymatic calorimetric test was used to measure the level of LDL-C.

Serum OPG concentration was measured by the enzyme-linked immunosorbent assay (ELISA; Oscotec, Korea) method from the serum samples of the subjects. The measurements were duplicated and the mean values were used as the detected level. The intra- and inter-assay coefficients of variance were 6.9-9.0% and 6.0-9.0%.

Measurements of bone turnover markers and hormones

In regard to the biochemical bone turnover markers, urine deoxypyridinoline was measured as the bone resorption marker (chemiluminescence immunoassay, ACS-180, CIBA-corning, Medfield, MA) and serum total alkaline phosphatase (ALP) was measured as the bone formation marker (total ALP, multiple-point rate colorimetric, Vitros 750 XRC, Ortho-Clinical Diagnostics, Rochester, NY). Urine calcium excretion was also measured by colorimetric method (Vitros 750
The detection of aortic calcification by simple radiological method

Calcification of either the thoracic or abdominal aorta was detected from simple posterior-anterior chest radiographs and lateral views of lumbar spine as described in previous aortic calcification studies [2,3]. The diagnosis of aortic calcification was made only when the radiological interpretation was agreed upon between two trained radiologists who had no detailed clinical information of the subjects.

Measurements of BMD

Dual energy X-ray absorptiometry (DXA, XR-series, Norland Instruments, Fort Atkinson, WI) was used to measure the BMD of the lumbar spine and the femoral neck. The lumbar spine BMD was measured from the 2nd to the 4th lumbar spine, and the femoral neck BMD was measured from the left femoral neck. Regarding the precision of the lumbar spine BMD, the coefficient of variation (CV) was 1.0%, and the CV of the femoral neck BMD was 1.2%. Osteoporosis and osteopenia were diagnosed according to the World Health Organization (WHO) definition: a T-value lower than –2.5 was diagnosed as osteoporosis and a T-value lower than –1.0 was diagnosed as osteopenia. T-scores for the lumbar spine and femoral neck were calculated on the basis of the standardized records of healthy Korean women.

Genotyping analysis of polymorphisms in the RANKL gene by 5’ nuclease polymerase chain reaction assay

Buffy coat was obtained from blood samples, frozen at -70°C, and genomic DNA was extracted from the preserved samples using Takara DNA Purification kits. The genotyping of the two polymorphisms in the RANKL gene was performed by allelic discrimination using the 5’ nuclease polymerase chain reaction assay. The detector used in this experiment was an ABI Prism 7200 sequence detection platform (Perkin Elmer). The primers used for genotype analyses were as follows;

- **rs2277438**
  - Forward primer: 5’CATCTTGAGGTTACATTAACTATTCA 3’
  - Reverse primer: 5’CTGCTATTTAATACAGTGGACTTAGAACAAA 3’
  - FAM-probe: 5’FAM - ACTCTTGCAAGTATG 3’
  - VIC-probe: 5’VIC - ACTCTTGCGAGTATGA 3’

- **rs9594782**
  - Forward primer: 5’CCATTTGACTTGCCGCTGAT 3’
  - Reverse primer: 5’AGTTGATTCACAACTTTAGTGGGATTCA 3’
  - FAM-probe: 5’FAM - CAAATAAATCAGCAGTTTT 3’
  - VIC-probe: 5’VIC - CAAATAAATCAGTAGTTTTT 3’

Statistical analysis

All data are presented as mean±standard deviation (SD). The comparisons of the mean values between different genotype groups in each RANKL polymorphisms were performed with independent-samples t-test and the associations between the presence of aortic calcification and RANKL polymorphisms were analyzed by χ²-test. The Hardy-Weinberg equilibrium for each genotype was analyzed with χ²-test. Binary logistic regression analysis was performed to compare the frequencies of aortic calcification after adjustment for age, BMI, blood pressure, fasting plasma glucose, serum HDL-C and LDL-C. Analysis of covariance (ANCOVA) was performed to compare the differences of urine deoxypyridinoline and serum HDL-C after adjustment for age and BMI. P value less than 0.05 was considered statistically significant.

Results

General characteristics of the study population

General characteristics of the study population are presented in Table 1. The baseline characteristic of this study population and the prevalence of aortic calcification classified according to age in this study population was also published previously [28].

The distribution of the genotypes of two polymorphisms in the RANKL gene

The frequency of rs2277438 polymorphism in the RANKL gene was 46.0% for AA, 41.4% for AG and
women, subjects with CT/CC genotypes of the rs9594782 polymorphism had 4.8 times higher risk of aortic calcification compared with TT genotype (95% CI, 1.21-18.68, \( p = 0.016 \)), and these significances disappeared after adjustment for age, BMI, blood pressure, fasting plasma glucose, serum HDL-C and LDL-C (\( p = 0.151 \)). In postmenopausal women, subjects with CT/CC genotypes of the rs9594782 polymorphism had 4.2 times higher risk of aortic calcification compared with TT genotype (95% CI, 1.15-15.40, \( p = 0.021 \)), and these significances persisted after adjustment for age, BMI, blood pressure, fasting plasma glucose, serum HDL-C and LDL-C (\( p = 0.014 \)). No significant associations were observed in analyses regarding rs 2277438 polymorphism (data not shown).

The differences of clinical variables according to the genotypes of RANKL gene polymorphisms

Among the two SNPs, rs9594782 polymorphism showed significant differences in the prevalence of aortic calcifications according to the minor allele, that is, subjects with CT/CC genotypes of the rs9594782 polymorphism had a 3.9 times higher risk of aortic calcification compared with TT genotype (95% CI, 1.57-9.59). This significance was persisted after adjustment for age, BMI, blood pressure, fasting plasma glucose, serum HDL-C and LDL-C (\( p = 0.001 \)) (Table 3).

When the subjects were divided into two groups according to the menopausal status, in premenopausal women, subjects with CT/CC genotypes of the rs9594782 polymorphism had 4.8 times higher risk of aortic calcification compared with TT genotype (95% CI, 1.21-18.68, \( p = 0.016 \)), and these significances disappeared after adjustment for age, BMI, blood pressure, fasting plasma glucose, serum HDL-C and LDL-C (\( p = 0.151 \)). In postmenopausal women, subjects with CT/CC genotypes of the rs9594782 polymorphism had 4.2 times higher risk of aortic calcification compared with TT genotype (95% CI, 1.15-15.40, \( p = 0.021 \)), and these significances persisted after adjustment for age, BMI, blood pressure, fasting plasma glucose, serum HDL-C and LDL-C (\( p = 0.014 \)). No significant associations were observed in analyses regarding rs 2277438 polymorphism (data not shown).

The differences of clinical variables according to the genotypes of RANKL gene polymorphisms

Mean levels of urine deoxypyridinoline were significantly higher in the subjects with AG/GG genotypes of the rs2277438 polymorphism compared with AA genotype (7.4±2.1 vs. 6.8±2.0 nMol/mMol, \( p = 0.043 \)). This difference was persistently significant even after adjustment for age and BMI with ANCOVA test.
RANKL polymorphisms and aortic calcification

RANKL polymorphisms and aortic calcification

ANCOVA test ($p=0.015$). Mean fasting plasma glucose level was significantly higher in the subjects with AG/GG genotypes of rs2277438 polymorphism compared with AA genotype (90.6±8.5 vs. 87.1±8.3 mg/dL, $p=0.045$), and this difference was persistently significant even after adjustment for age and BMI with ANCOVA test ($p=0.032$).

No significant differences were observed in mean values of the parameters in premenopausal women regarding rs2277438 polymorphism (data not shown).

For rs9594782 polymorphism, mean serum HDL-C level was significantly lower in subjects with CT/CC genotypes of rs9594782 polymorphism compared with TT genotype in premenopausal women (55.6±9.7 mg/dL vs. 60.4±14.7 mg/dL, $p=0.029$), but these differences were lost after adjustment for age and BMI with ANCOVA test ($p=0.118$).

There were no differences in mean values for age, BMI, blood pressure, fasting plasma glucose, serum total cholesterol, triglyceride, LDL-C, calcium, total ALP, OPG, urinary calcium excretion, and BMD values according to different genotypes of each polymorphism in the RANKL gene (Table 4).

When the analyses were performed in two groups according to the menopausal status, in postmenopausal women, mean levels of urine deoxypyridinoline were significantly lower in the subjects with CT/CC genotypes of rs9594782 polymorphism compared with TT genotype (7.1±2.0 vs. 7.1±2.1, $p=0.092$), but these differences were lost after adjustment for age and BMI with ANCOVA test ($p=0.032$).

No significant differences were observed in mean values of the parameters in premenopausal women regarding rs9594782 polymorphism (data not shown).

For rs9594782 polymorphism, mean serum HDL-C level was significantly lower in subjects with CT/CC genotypes of rs9594782 polymorphism compared with TT genotype in premenopausal women (55.3±5.2 vs. 60.9±13.5 mg/dL, $p=0.003$), and this difference disappeared after adjustment for age and BMI with ANCOVA test ($p=0.092$). No significant differences were observed in mean values of the parameters in premenopausal women regarding rs9594782 polymorphism (data not shown).

### Table 4  The clinical variables according to different genotypes of two polymorphisms in the RANKL gene

<table>
<thead>
<tr>
<th>Genotype</th>
<th>rs2277438</th>
<th>rs9594782</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AA (n=109)</td>
<td>AG+GG (n=128)</td>
</tr>
<tr>
<td>Age (year)</td>
<td>51.1±6.7</td>
<td>51.5±7.1</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.1±2.9</td>
<td>23.9±2.9</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>123.9±18.3</td>
<td>124.9±18.3</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>74.8±11.8</td>
<td>75.5±12.4</td>
</tr>
<tr>
<td>FPG (mg/dL)</td>
<td>87.1±8.7</td>
<td>89.2±10.8</td>
</tr>
<tr>
<td>TC (mg/dL)</td>
<td>197.4±34.9</td>
<td>200.6±38.8</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>107.8±53.1</td>
<td>119.8±61.3</td>
</tr>
<tr>
<td>HDL-C (mg/dL)</td>
<td>59.9±14.6</td>
<td>59.7±14.0</td>
</tr>
<tr>
<td>LDL-C (mg/dL)</td>
<td>115.9±31.6</td>
<td>116.9±35.3</td>
</tr>
<tr>
<td>Calcium (mg/dL)</td>
<td>9.0±0.4</td>
<td>9.0±0.3</td>
</tr>
<tr>
<td>Total ALP (IU/L)</td>
<td>58.3±19.6</td>
<td>60.9±18.2</td>
</tr>
<tr>
<td>DPD (nMol/mMol)</td>
<td>6.8±2.0*</td>
<td>7.4±2.1*</td>
</tr>
<tr>
<td>Urine Ca/Cr</td>
<td>0.218±0.118</td>
<td>0.229±0.132</td>
</tr>
<tr>
<td>OPG (ng/mL)</td>
<td>1.3±0.4</td>
<td>1.3±0.4</td>
</tr>
<tr>
<td>LS BMD (g/cm²)</td>
<td>0.949±0.162</td>
<td>0.959±0.174</td>
</tr>
<tr>
<td>FN BMD (g/cm²)</td>
<td>0.795±0.122</td>
<td>0.805±0.121</td>
</tr>
<tr>
<td>TSH (mIU/L)</td>
<td>2.0±1.4</td>
<td>1.9±1.1</td>
</tr>
<tr>
<td>FSH (IU/L)</td>
<td>38.2±33.2</td>
<td>43.1±30.6</td>
</tr>
</tbody>
</table>

Data are given as mean values±SDs.

Abbreviations: BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; FPG, fasting plasma glucose; TC, total cholesterol; TG, triglyceride; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; ALP, Alkaline Phosphatase; DPD, Deoxypyridinoline; Ca/Cr, Calcium to Creatinine Ratio; OPG, Osteoprotegerin; LS BMD, lumbar spine bone mineral density; FN BMD, femoral neck bone mineral density; TSH, thyroid stimulating hormone; FSH, follicle stimulating hormone.

The comparisons of the mean values for variables between genotypes were performed with independent-samples $t$-test. *$p<0.05$ in comparison analysis between genotypes.
Discussion

To our knowledge, this is the first study showing the association of RANKL gene polymorphisms with aortic calcification in human. In this study performed in apparently healthy women, subjects with C allele in rs9594782 polymorphism in RANKL gene had 3.9 times increased risk for aortic calcification compared with those with TT genotypes, and mean urine deoxypyridinoline level, a bone resorption marker, was significantly higher in subjects with G allele in rs2277438 polymorphism in RANKL gene compared with those with AA genotypes. These results suggest the role of RANKL gene polymorphism in relation to vascular calcification and bone metabolism in humans.

Vascular calcification is a pathological sequence of events that has similarities to the normal physiological process of osteogenesis [29]. It is thought to result from an imbalance in both local and systemic inhibitors and promoters [30]. Many animal models with targeted deletion of a single gene leading to combined phenotype of osteoporosis and vascular calcification, such as, mice deficient in fetuin-A, an important circulating calcification inhibitor [31], mice deficient in Klotho, a regulator of phosphate homeostasis [32]. In recent years, RANKL/OPG signaling pathway is established to be central to the processed regulating bone turnover and vascular calcification [7]. The core of the mechanism linking RANKL/OPG system in the relationship between vascular calcification and osteopenia is the modulatory role it has on mineralization in bone and vasculature through the handling of osteoclastogenesis [20].

Until recently, the evidence linking RANKL with vascular calcification has been circumstantial and indirect. A recent study by Panizo et al. report direct links between RANKL/RANK signaling, elevated levels of BMP4 and increased vascular calcification [21]. They demonstrated that the addition of RANKL to VSMC in vitro accelerates mineralization and that in an in vivo model, the increase in vascular calcium content was parallel to an increase in RANKL and BMP4 expression, both through activation of alternative NF-κB pathway. In a very recent study by Helas et al., RANKL inhibition by denosumab, a human monoclonal antibody against RANKL reduced vascular calcium deposition in glucocorticoid-induced osteoporosis in mice, which could be considered as a direct evidence for the link between the bone and vascular system [33].

There are several candidate genes that may in part explain the possible relationships between vascular calcification and bone metabolism in humans [29]. Gene polymorphisms in KLOTHO, which encodes a hormone with anti-aging properties that is involved in phosphate regulation, were reported to be associated with bone mineral density and coronary artery disease in Asian population [34-36]. Polymorphisms in OPG gene, which is a decoy receptor for RANKL, were reported to be associated with osteoporosis and increased risk of coronary artery disease and vascular morphology [22, 37]. In a study performed in the same study population as this study, no significant relationship was found between four OPG gene polymorphisms and aortic calcification or cardiovascular risk factors [27]. Other existing polymorphism studies performed in relation to vascular calcification and bone metabolism are MGP and osteopontin [38].

In this study, we demonstrated for the first time in the literature, the possible relationship of two RANKL gene polymorphisms, rs9594782 and rs2277438, with aortic calcification in female subjects. However, there were no significant associations of these two polymorphisms with BMD values in this population, despite that rs2277438 polymorphism showed a significant association with only urine deoxypyridinoline levels. For rs9594782 polymorphisms, C allele carriers showed 3.9 times increased risk for having aortic calcification compared with those with TT genotype, suggesting the possible involvement of this polymorphism in the calcification process in human body. Although there are no studies performed on the association of these polymorphisms with vascular calcification, in a previous study by Hsu et al., C allele carriers in rs9594782 polymorphism in RANKL gene showed a 2.1 times higher risk of extremely low hip BMD and lower whole body BMD in Chinese population [26]. Although the results from the previous study and our study show discrepancy in that BMD values failed to show significant associations with BMD values in this population, our study finding has a meaning in that this was the first study to report the association of RANKL polymorphisms with aortic calcification in human, warranting further need for extended studies in different ethnic groups.

Our study has limitations. Firstly, as the polymorphisms failed to show significant association with BMD values, significant functional role of RANKL gene in the association of vascular calcification and
bone metabolism, could not be confirmed. Although the association could be assumed from the cellular mechanism that RANK/RANKL system has on bone and vessel, direct relationship has to be confirmed from further studies reproducing our study results. Secondly, since potential functions of these polymorphisms are not significantly clarified from the cellular studies, the interpretation of the results has to be very careful. It is also possible that this polymorphism in linkage disequilibrium with the real causal genetic variation in the RANKL gene or in a nearby gene. Thirdly, the sample size of the study populations is relatively small for a genetic association study. Therefore, further studies with large population number in different groups with different characteristics are needed.

Conclusions

In conclusion, the results of this study show that a

Rs9594782 showed significant association with increased risk for aortic calcification in Korean female subjects, and another polymorphism rs2277438 showed a significant association with urine deoxypyridinolone level, a bone resorption marker. These results are the first to show the association of RANKL gene polymorphism with aortic calcification in humans, suggesting for further extensive studies to draw a clear conclusion.

Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References


