



## Polymorphisms within RANKL and Osteoprotegerin Genes in Low Bone Mass among Postmenopausal Indonesian Women

Postmenopozal Endonezyalı Kadınlarda Düşük Kemik Kütlesindeki RANKL ve Osteoprotegerin Genlerindeki Polimorfizmler

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### Abstract

**Objective:** Prior studies have shown that receptor activator of nuclear factor- $\kappa$ B ligand (RANKL) and osteoprotegerin (OPG) have an essential role in bone remodeling and osteoporosis. This study aimed to investigate the association between RANKL (TNFSF11) and OPG (TNFRSF11B) genes' polymorphisms with low bone mineral density (BMD) in Indonesian postmenopausal women.

**Materials and Methods:** Sixty postmenopausal women aged between 50-65 years were eligible. The BMD was measured by using dual-energy X-ray absorptiometry. The genotypes of TNFSF11 and TNFRSF11B were obtained by polymerase chain reaction-restriction fragment length polymorphisms and DNA sequencing methods. Three single nucleotide polymorphisms (SNPs) from TNFSF11 (-290C>T, -643C>T, -693G>C) and from TNFRSF11B (163A>G, 950T>C, 1181G>C) were selected. The association between alleles distribution and BMD was computed using chi-square or Fischer's exact test. Multiple logistic regression was used to analyze between all SNPs and BMD at bone sites. Significance was set at  $p < 0.05$ .

**Results:** Most subjects had lower BMD (83.3%). Characteristics of subjects between healthy and low BMD were comparable (all  $p > 0.05$ ). The distribution of genotypes and alleles in TNFSF11 and TNFRSF11B between healthy and low BMD were not significantly different (all  $p > 0.05$ ). There was no association between SNPs of TNFSF11 and TNFRSF11B with BMD at all bone sites (all  $p > 0.05$ ).

**Conclusion:** The present study suggests that TNFSF11 and TNFRSF11B gene polymorphisms are not associated with BMD in Indonesian postmenopausal women aged between 50-65 years old.

**Keywords:** Bone mineral density, RANKL gene, OPG gene, polymorphism, postmenopause

### Öz

**Amaç:** Reseptör aktivatör nükleer faktör- $\kappa$ B ligandı (RANKL) ve osteoprotegerinin (OPG) kemik yeniden modelleme ve osteoporozda önemli bir rolü olduğu gösterilmiştir. Bu çalışmada, Endonezya menopoz sonrası kadınlarda düşük kemik mineral yoğunluğu (KMY) ile RANKL ve OPG genlerinin polimorfizmleri (sırasıyla TNFSF11 ve TNFRSF11B) arasındaki ilişkinin araştırılması amaçlanmıştır.

**Gereç ve Yöntem:** Elli - altmış beş yaş arası 60 postmenopozal kadın uygun bulundu. KMY, çift-enerjili X-ışını absorpsiyometrisi kullanılarak ölçülmüştür. TNFSF11 ve TNFRSF11B'nin genotipleri polimeraz zincir reaksiyonu-kısıtlama fragman uzunluğu polimorfizmleri ve DNA dizileme yöntemleri ile elde edildi. TNFSF11'den (-290C> T, -643C> T, -693G> C) ve TNFRSF11B'den (163A>G, 950T>C, 1181G>C) üç tek nükleotid polimorfizmleri (SNP) seçildi. Allel dağılımı ile KMY arasındaki ilişki ki-kare veya Fischer kesin testi kullanılarak hesaplandı. Kemik bölgelerindeki tüm SNP'leri ve BMD'yi analiz etmek için çoklu lojistik regresyon kullanıldı. Anlamlılık  $p < 0,05$  olarak ayarlandı.

**Bulgular:** Çoğu birey daha düşük KMY'ye (%83,3) sahipti. Sağlıklı ve düşük KMY arasındaki bireylerin özellikleri karşılaştırılabilir bulundu (hepsi  $p > 0,05$ ). TNFSF11 ve TNFRSF11B'de genotiplerin ve allellerin sağlıklı ve düşük KMY'ler arasındaki dağılımı anlamlı olarak farklı değildi (hepsi  $p > 0,05$ ). Tüm kemik bölgelerinde TNFSF11 ve TNFRSF11B'nin SNP'leri ile KMY arasında ilişki yoktu (hepsi  $p > 0,05$ ).

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**Sonuç:** Bu çalışma, 50-65 yaşlarındaki Endonezyalı postmenopozal kadınlarda TNFSF11 ve TNFRSF11 gen polimorfizmlerinin BMD ile ilişkili olmadığını düşündürmektedir.

**Anahtar kelimeler:** Kemik mineral yoğunluğu, *RANKL* geni, *OPG* geni, polimorfizm, postmenopoz

## Introduction

The prevalence of low bone mass worldwide is high and varies between countries. In industrialized countries, the prevalence of osteoporosis ranged from 9% to 38% (1). In developing countries, prevalence of osteoporosis is comparable. The data from Indonesian Osteoporosis Society in 2007 showed that the number of elderly women with osteopenia in Indonesia was 90% and osteoporosis was about 32.3% of the total of the Indonesian population (2). The Indonesian National Statistics Bureau and the Indonesian Ministry of Health reported that the number of women with osteopenia and osteoporosis accounts for 9.6 million and 2.2 million in 2012-2013 (3,4). This number will continue to increase as prevalence of osteoporosis worldwide increases.

Low estrogen level plays an important role in the pathophysiology of low bone mineral density. Estrogen affects osteoclast and osteoblast activities, notable cells that regulate bone remodeling process. Estrogen increases osteoblast activity resulting in bone formation, whereas low estrogen increases osteoclast activity resulting in bone resorption (5). At the molecular level, bone remodeling involves receptor activator of nuclear factor- $\kappa$ B ligand (RANKL, a membrane protein and a member of tumor necrosis factor [TNF] superfamily), RANK (osteoclast cell-surface receptor that binds to RANKL), and osteoprotegerin (OPG, a TNF superfamily and competitive inhibitor of RANK). RANKL is expressed by osteoblast on the cell surface. Formation of the RANK-RANKL complex initiates activation and maturation of osteoclasts, leading to bone resorption. Otherwise, the formation of the OPG-RANKL complex inhibits activation and maturation of osteoclast, inducing bone formation (6,7). Results from prior studies showed estrogen improved OPG production and accelerated osteoclast apoptosis. Therefore, lack of estrogen can attenuate bone formation and increase bone resorption resulting in low bone mass (8,9).

Specific genes encode RANKL and OPG and are associated with osteoporosis genetic susceptibility (10,11). TNF ligand superfamily member 11 (TNFSF11) gene encodes RANKL while TNF receptor superfamily member 11B (TNFRSF11B) gene encodes OPG (12,13). Gene polymorphisms in osteoporosis are strongly influenced by race and ethnicity and are related to bone density on different skeletal sites (14,15). A study in Poland reported -643C>T *RANKL* gene was associated with osteoporosis development whereas study in Slovak found 245T>G *OPG* gene increased risk of osteoporotic fractures (16,17). Sassi et al. (18) demonstrated the relationship between -643 *RANKL* gene and osteoporosis risk in postmenopausal Tunisian women. No studies have been carried out on the TNFSF11 and TNFRSF11B gene polymorphisms and their association with BMD sites in the Indonesian population. Therefore, the purpose of this study is

to identify the polymorphism and its relationship with BMD at different skeletal sites in postmenopausal women in Indonesia.

## Materials and Methods

### Subjects

A cross-sectional study involved sixty women according to the eligibility criteria: female, age between 50–65 years, experiencing postmenopausal symptoms for at least 12 months, and Indonesian native. Participants were excluded if they used medication affecting bone mass, smoked, drank alcohol, or had a disease associated with low BMD (thyroid disease, chronic renal failure, diabetes mellitus, etc.). There was no family relationship among the participants.

All study participants agreed to be involved in the study by signing informed consent forms. The study was approved by the Research Ethics Committee of the Faculty of Medicine, Universitas Indonesia and Cipto Mangunkusumo Hospital (Protocol number: 679/UN2.F1/ETIK/2014). All procedures of this study were implemented according to the Declaration of Helsinki guidelines.

### Measurements

Measurements of height and weight were performed. Height was measured using a wall-mounted stadiometer in the standing position without footwear. Height was reported in cm with 0.1 cm precision. Body height was measured using a portable digital scale (SECA, Robusta 813, Germany) with subjects in minimal clothes. Weight was reported in kg with 0.1 kg precision. Body mass index ( $\text{kg}/\text{m}^2$ ) is calculated by dividing weight (kg) with height (m) square.

### Dual X-ray Absorptiometry and Radiography Examination

Bone mineral density was assessed using a standard DXA (Lunar Prodigy DF $\pm$ 301251, GE Healthcare, USA). The equipment was regularly calibrated. The DXA examination was performed by experts according to the standard procedure from the International Society for Clinical Densitometry. Bone mass density of the bone sites most susceptible to fracture were presented. Bone mineral density was expressed in grams per  $\text{cm}^2$  ( $\text{g}/\text{cm}^2$ ). According to the category by the WHO for BMD results, participants were classified as normal if T score > -1 SD, osteopenia if T score between -1 and -2.4 SD, and osteoporosis if T score  $\leq$ -2.5 SD (19). For statistical purposes, osteopenia and osteoporosis were combined in one group denominated as "low BMD". Subjects were diagnosed as "low BMD" if they had low BMD at least on one site.

## Genotyping

Genomic DNA was obtained by extracting peripheral blood leucocytes using salting-out methods (20). The presence of TNFSF11 and TNFRSF11B gene was obtained from genbank (Acc No. NG\_008990 for TNFSF11, Acc No. NO\_012202 for TNFRSF11B). Three SNPs from TNFSF11 gene were selected as reported by previous studies: SNPs -290C>T, -643C>T and -693G>C; and three SNPs from TNFRSF11B gene: SNPs 163A>G, 950T>C, and 1181G>C (21-23). The SNPs was identified using BioEdit software v7.0.0. Primers of TNFSF11 gene (SNPs -290C>T, -643C>T, and -693G>C) and TNFRSF11B gene (SNPs 163A>G, 950T>C, and 1181G>C) are listed in Table 1.

The genotype was screened using polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP). The PCR-RFLP was performed using the KAPA Tag Extra Hostart PCR kit® mix (KAPA Biosystem, USA). A total of 25 µL PCR reactions were performed using 0.4 pmol/L primers, 3.5 mM MgCl<sub>2</sub> 0.4 mM dNTP Mix, and 0.05 units/µL. The RFLP was carried out by using specific restriction enzymes for the digestion process of PCR products. The digestion process was completed by adding 6 units of specific restriction enzymes and then incubated at a certain temperature and time. The restriction enzymes, temperature, and duration of incubation for all SNPs were summarized in Table 1. Sequencing was carried out following RFLP to confirm that DNA fragment was in accordance with the expected fragment.

## Statistical Analysis

Numerical data were presented as mean ± standard deviation (SD), while categorical data as frequencies and percentage (%). Results of BMD were presented in g/cm<sup>2</sup> and according

to the t-score. Chi-square or Fischer exact test was used to analyze distribution of the allele between healthy and low BMD. Multivariate logistic regression was applied to analyze the association between SNPs and BMD of each bone sites. A p value <0.05 was considered significant. The statistical analysis was performed using the SPSS ver. 17.0 (SPSS Inc., Chicago, IL, USA).

## Results

Characteristics of the participants consisted of age, weight, height, and BMI, as presented in Table 2. Bone mineral density of four bone sites (L1-L4, femoral neck, total femur, and third distal radius) were selected as most vulnerable sites to fracture. Fifty subjects (83.3%) were diagnosed as having low BMD in which most of them (82%) had low BMD on more than one bone site (82%). Low BMD at single site was found on lumbar and radius.

Genotype and allele frequency of the three SNPs in the RANKL and OPG gene in healthy and low BMD subjects are shown in Table 3. Genotype homozygous dominant tends to be more prominent in TNFRSF11B whereas genotype heterozygous and homozygous recessive in TNFSF11. However, none of the distributions of the genotypes and allele in the RANKL and OPG genes were significantly different (all p>0.05).

Further analysis was performed to examine the correlations between BMD of bone sites and SNPs of genes using a multivariate logistic regression. The BMD of bone sites had no correlation with each SNPs in both genes (all p>0.05). Adjusted regression showed no correlation between BMD of bone sites and all SNPs of both genes (all p>0.05) (Table 4).

**Table 1. Procedures of genotyping for TNFSF11 and TNFRSF11B polymorphisms**

SNPs	Direction	Primers	Restriction enzyme	Incubation period/temperature
TNFSF11 gene				
SNP-290C>T	Forward	GGGAGAGGTTGGACAGGAAG	DraI	16 hours/37 °C
	Reverse	GTCCTTCTGGAGGTCCAAGAGATGGTTTAA		
SNP-643C>T	Forward	TGGTCAGCAACTTCCTTCTG	TspRI	3 hours/65 °C
	Reverse	GACATTCCTCCTGCATCCAT		
SNP-693G>C	Forward	TGGTCAGCAACTTCCTTCTG	BsaI	3 hours/60 °C
	Reverse	GACATTCCTCCTGCATCCATT		
TNFRSF11B gene				
SNP-163A>G	Forward	CTGGAGACATATAACTTGAACA	AseI	3 hours/37 °C
	Reverse	CCATCATCAAAGGGCTATTGGT		
SNP-950T>C	Forward	GTTCTCAGCCCGGTGGCTTT	HpaI	3 hours/37 °C
	Reverse	TGTGGTCCCGGAAACCTCAGG		
SNP-1181G>C	Forward	ACTTCTGTTGCCGGGACGCTA	SmaI	3 hours/55 °C
	Reverse	GGACTTACCACGAGCGCGCAGCACCTCA		

SNP: Single nucleotide polymorphisms, TNFSF11: Tumor necrosis factor ligand superfamily member 11, TNFRSF11B: Tumor necrosis factor receptor superfamily member 11B

## Discussion

Polymorphisms in low BMD have been investigated worldwide. However, due to the possible influence of race and ethnic factors, new studies on polymorphisms in osteoporosis will still be fascinating and useful. This might be the first study on polymorphism in low BMD in Indonesian women. The results indicated that polymorphisms of the TNFSF11 and TNFRSF11B genes were not associated with low BMD in Indonesian postmenopausal women. This study also did not find any association between SNPs and alleles with low BMD.

**Table 2. Characteristics of the participants and bone mineral density**

Characteristics	Mean ± SD
Age (years)	57.9±5.31
Weight (kg)	57.4±9.31
Height (cm)	149.9±4.46
BMI (kg/m <sup>2</sup> )	25.5±3.80
BMD (g/cm <sup>2</sup> )	-
L1-L4	0.91±0.15
Femoral neck	0.75±0.15
Total femur	0.82±0.14
3 <sup>rd</sup> distal radius	0.67±0.13
BMD (T score)	n (%)
Normal	10 (16.7%)
Low	50 (83.3%)
-Radius	7 (14%)
-L1-L4	2 (4%)
->1 sites	41 (82%)

BMD: Bone mineral density, BMI: Body mass index, L: Lumbar

Polymorphisms on low BMD has been investigated but the results was less consistent between studies. Race or ethnic, SNPs types, and bone sites might contribute to this. Prior studies on osteoporosis gene polymorphisms had been conducted in postmenopausal women from different race and ethnic including Caucasian, Hispanic and Asian. A study by Vidal et al. (24) involved postmenopausal Caucasian women in Malta and found an association between OPG genes and low BMD. A meta-analysis study by da Silva et al. (25) also found an association between low BMD and SNPs of 163A>G, 950T>C and 1181G>C (25). The study of polymorphisms was also conducted in Asia largely in China and Korea (26-29). A study by Sun et al. (26) involving postmenopausal Chinese women suggested that OPG genetic polymorphisms may affect BMD and osteoporosis. Study in postmenopausal Korean women by Kim et al. (28) indicated that OPG gene polymorphisms were related to BMD and osteoporosis. The influence of race and ethnicity on osteoporosis gene polymorphisms was also supported by Wang et al. (30) which investigated RANKL/RANK/OPG gene polymorphisms and involved five different races and ethnicities including China, Japan, Mongol, African, and Caucasian. Polymorphisms on RANKL/RANK/OPG gene were different among races and ethnic groups. Information on gene polymorphisms in osteoporosis from other race and ethnics are needed. Our study in Indonesian women showed that OPG and RANKL gene polymorphisms had no association with low BMD in postmenopausal women. However, results of our study less conclusive and need further study with larger sample and SNPs.

OPG, RANK, and RANKL gene are the most frequent genes investigated to elucidate the role of polymorphisms in osteoporosis. Prior studies indicated that not all of these gene polymorphisms are proven to be related with BMD

**Table 3. The association between allele frequencies and BMD**

TNFSF11	Genotypes	Healthy	Low BMD	Allele	Healthy	Low BMD	p
SNP -290C>T	CC	1 (10%)	17 (34%)	C	10	63	0.28*
	CT/TT	9 (90%)	33 (66%)	T	10	37	
SNP -643C>T	CC	2 (20%)	12 (24%)	C	11	60	0.62*
	CT/TT	8 (80%)	38 (76%)	T	9	40	
SNP -693G>C	GG	2 (20%)	10 (20%)	G	10	54	0.74*
	GC/CC	8 (80%)	40 (80%)	C	10	46	
TNFRSF11B							
SNP 163A>G	AA	7 (70%)	36 (72%)	A	17	85	1.00+
	AG/GG	3 (30%)	14 (28%)	G	3	15	
SNP 950T>C	TT	7 (70%)	29 (58%)	T	17	78	0.76+
	TC/CC	3 (30%)	21 (42%)	C	3	22	
SNP 1181G>C	GG	6 (60%)	21 (42%)	G	10	69	0.10*
	GC/CC	4 (40%)	29 (58%)	C	10	31	

SNP: Single nucleotide polymorphisms, BMD: Bone mineral density, TNFRSF11B: Tumor necrosis factor receptor superfamily member 11B, TNFSF11: Tumor necrosis factor ligand superfamily member 11, \*Chi square, +Fischer exact test

**Table 4. The correlation between BMD of bones sites and SNPs**

TNFSF11	SNP -290C>T		SNP -643C>T		SNP -693G>C		Adjusted R	p
	$\beta$	p	$\beta$	p	$\beta$	p		
L1-L4	0.31	0.08	-0.26	0.22	0.06	0.72	0.00	0.38
Femoral neck	0.22	0.21	-0.02	0.93	0.10	0.54	0.02	0.27
Total femur	0.21	0.25	-0.17	0.43	0.03	0.87	-0.03	0.72
3 <sup>rd</sup> distal radius	0.30	0.10	-0.36	0.09	0.06	0.73	0.01	0.30
TNFRSF11B	SNP 163A>G		SNP 950T>C		SNP 1181G>C		Adjusted R	p
	$\beta$	p	$\beta$	p	$\beta$	p		
L1-L4	0.04	0.76	0.01	0.94	-0.21	0.13	-0.01	0.47
Femoral neck	-0.05	0.71	-0.03	0.82	-0.01	0.93	-0.05	0.97
Total femur	-0.12	0.37	-0.08	0.56	0.01	0.94	-0.03	0.74
3 <sup>rd</sup> distal radius	0.07	0.60	0.07	0.59	0.10	0.45	-0.03	0.68

BMD: Bone mineral density, L: Lumbal, SNP: Single nucleotide polymorphisms, TNFRSF11B: Tumor necrosis factor receptor superfamily member 11B

and osteoporosis although in the same race and ethnicity. Two meta-analysis studies confirmed the link of OPG gene polymorphisms with BMD and osteoporosis (31,32). A study by Kim et al. (28) showed that among OPG, RANK, and RANKL which were investigated, only OPG had an association with BMD and osteoporosis. A similar result was reported by Shang et al. (33) which involved peri- and postmenopausal women in China. The findings suggested that OPG and RANK but not RANKL genetic polymorphisms influenced BMD in Chinese women. An opposite result was reported by Tu et al. (34) their study illustrated that RANK and RANKL but not OPG gene influence BMD in Chinese postmenopausal women. The recent study examined the association between RANKL and OPG gene polymorphisms, and no association was found. From the results of the studies, which gene polymorphisms are most associated with BMD have not been determined yet and require further confirmation from future studies.

Alleles and genotypes in genes determine the influence of gene polymorphisms on BMD with the effect could be positive or negative. Two meta-analysis study found G allele of the OPG 163A>G polymorphisms increased osteoporosis risk in Caucasians whereas allele C of the OPG 1181G>C decreased osteoporosis risk mainly in Asians (31,32). A pilot study by Cvijetic showed that postmenopausal women with osteoporosis had AG genotype (of the 163A>G) polymorphism more frequently than women without osteoporosis while the prevalence of TT and GG polymorphism between patients with and without osteoporosis was not significantly different (35). Langdahl et al. (23) reported that in SNP 163A>G, G alleles were more commonly found in subjects with osteoporosis than normal. GG genotypes are also more commonly found in subjects with osteoporosis whereas, in SNP 950T>C, CC genotype was higher in the normal group than in the osteoporosis group (23). Association of 950T>C and osteoporosis was also reported by Li et al. (36) using a meta-analysis study in Chinese. A study by Sun et al. (26) found subjects with genotype TT of the

g.26395T>C and genotype AA of the g.27649A>G genetic polymorphism had a significantly higher BMD when compared with those of genotypes TC and CC and those of genotypes AG and GG, respectively. Our study did not find any association between allele and genotype with BMD but G1181C of OPG genes showed the smallest p value (0.1) in which GG genotype and G allele were more pronounced in low BMD while GC/CC genotype and C allele were higher in normal. Alleles seems to be more influential on BMD than SNPs in which G allele is associated with susceptibility to low BMD whereas C allele had a protective effect against low BMD.

The influence of gene polymorphisms might be specific to bone sites. Piedra et al. (37) reported a relationship between the genotype of SNPs and BMD in patients with primary hyperparathyroidism. Minor homozygote genotype (GG for 163A>G) had significantly lower BMD in 1/3 distal radius but not for GG for 1181G>C. A study by Tu et al. (34) also illustrated the relationship between polymorphisms and bone sites. The results confirmed that RANK and RANKL gene polymorphisms affect BMD in the femoral neck. Our study found no association between SNP and any bone sites. Analysing distribution of low BMD in each bone site, most of the subjects had low BMD on more than one bone site while there were only little low BMD on single bone site in our subjects. Also, the number of subjects with normal BMD was too low. These may cause a lack of significant association between SNPs and bone sites.

Several risk factors are attributed to low bone mass. Age at menarche, age at menopause, older age, height and BMI have been recognized as risk factors for low BMD (38-40). Age at menarche and at menopause had been known to have a relationship with osteoporosis gene polymorphisms (RANK gene) (41). Influence of other risk factors (age, height, BMI) on polymorphisms in low BMD is not known and thus requires an advanced study.

The important limitation of this study is sample size and SNPs frequency. The study on genomic and polymorphisms requires



a large number of subjects and SNPs. A small sample and SNPs result in a decrease of statistical power to identify small differences between groups that may exist in this study. Also, the distribution of BMD value between the groups was not comparable. Only 16.7% of subjects had normal BMD. In addition, as there are many ethnicities and tribes in Indonesia, it is recommended to conduct further studies with larger sample sizes and SNPs among various ethnicities and tribes to obtain a better result.

## Conclusion

In summary, this preliminary study with small sample size did not find any association between OPG and RANK gene polymorphisms with low BMD in Indonesian postmenopausal women aged 50–65 years.

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## Ethics

**Ethics Committee Approval:** The study was approved by the Research Ethics Committee of the Faculty of Medicine, Universitas Indonesia and Cipto Mangunkusumo Hospital (Protocol number: 679/UN2.F1/ETIK/2014).

**Informed Consent:** All study participants agreed to be involved in the study by signing informed consent forms.

**Peer-review:** Externally peer-reviewed.

## Authorship Contributions

Concept: I.R.H., Design: I.R.H., H.S., E.I.I., S.W.J., A.P., Data Collection or Process: I.R.H., M.A., Analysis and Interpretation: M.A., I.R.H., E.I.I., A.L., Literature Research: I.R.H., A.P., A.T., S.W.J., H.S., Writing: I.R.H.

**Conflict of Interest:** The authors declare that there is no conflict of interest in any form.

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