OSTEOPROTEGERIN (T950C) POLYMORPHISM AND OSTEOPOROSIS IN POSTMENOPAUSAL INDONESIAN WOMEN

[1]AUERKARI EI, [2]SUSIANA E, [3]SURYANDARI DA, [4]KUSDHANY L, [5]RAHARDJO TBW

[1] Department of Oral Biology, Faculty of Dentistry, University of Indonesia, Jakarta
[1,5]Centre for Ageing Studies, University of Indonesia, Depok, Indonesia
[2,3,4]Department of Medical Biology, Faculty of Medicine, University of Indonesia, Jakarta
[4]Department of Prosthodontics, Faculty of Medicine, University of Indonesia, Jakarta
E-mail: eauerkari@yahoo.com; elza.ibrahim@ui.ac.id

Abstract - Introduction: Bone remodeling is controlled by the balance of bone formation and resorption, where osteoprotegerin (OPG) has a role in inhibiting bone resorption by osteoclasts. In postmenopausal women, the balance is tipped towards resorption, resulting in bone loss and finally osteoporosis. This study aimed to explore the possible association of the OPG gene (T950C) polymorphism with bone mineral density (BMD) and osteoporosis risk in postmenopausal Indonesian women.
Materials and methods: The study included 285 consenting postmenopausal Indonesian women, of which 81 were classified as normal (healthy), 143 with osteopenia, and 61 with osteoporosis according to T-scores based on ultrasound densitometry of calcaneous bone. Blood-derived DNA samples were used for PCR-RFLP analysis of OPG polymorphism (T950C, rs2073617). Chi-square and OR testing were mainly used for statistical analysis, assuming significance at $p < 0.05$.
Results: The results showed a genotype (TT, TC, CC) distribution consistent with the Hardy-Weinberg equilibrium but with low fraction (3.2%) of the mutant CC genotype compared to the same polymorphism reported from elsewhere. No significant difference of the genotype or allele frequencies was found in the BMD groups, and it was concluded that the tested polymorphism is not associated with the risk of osteoporosis.

Key words - Osteoporosis, OPG, polymorphism, T950C, rs2073617

I. INTRODUCTION

In the process of remodeling by osteoblast and osteoclast function, bone is throughout life experiencing continuous formation and resorption, and osteoporosis can result from an imbalance where the latter is faster. Menopause, usually at the age 45 to 52 years, is a period of transition to declining ovarian function and reduced estrogen levels that lead to increasing incidence of osteopenia and osteoporosis.[1-4] Soon after menopause, bone mass (bone mineral density, BMD) can decrease at a rate of 3-5% per year, increasing the associated risk of fracture.[5]

Bone metabolism involves complex regulatory networks, but is strongly affected by three proteins, osteoprotegerin (OPG), receptor activator of nuclear factor κB (RANK) and receptor activator of nuclear factor κB ligand (RANKL) that have essential roles in the maintenance of bone tissue. Activation of osteoclasts for increased resorption will require interaction of RANKL and RANK, while OPG acts as a soluble decoy receptor binding to RANKL, thereby inhibiting RANKL-mediated osteoclastogenesis, osteoclast action and bone resorption.[4-7] OPG is a glycoprotein cytokine member of the tumor necrosis factor (TNF) superfamily, encoded by the corresponding OPG (TNFRSF11B) gene at the human chromosomal position 8q24. Lacking expression of OPG in animal models results in severe loss of bone mass, i.e. osteoporosis.[8] Therefore genetic variation such as OPG gene polymorphism could also affect the likelihood or severity of osteoporosis. However, OPG is secreted by many cell types and is widely present in the body, and for example reduced BMD may or may not be reflected in the serum levels of OPG.[2,9,10] Also, association studies comparing frequencies of OPG polymorphisms with the measured BMD and occurrence of osteoporosis have frequently found no association or produced contradictory results depending on the tested polymorphism, population and applied methods.[11-21]

Partly to clarify the mixed results and contradictions in the indicated OPG role, this study aims to assess the association of a OPG polymorphism with the risk of osteoporosis, with particular emphasis in postmenopausal Indonesian women. The selected single nucleotide polymorphism (SNP) is T950C that resides in the OPG promoter, 223 bp upstream of the translation initiation site in the OPG 5' untranslated region.

II. MATERIALS AND METHODS

In total, 285 consenting postmenopausal women from the East Bekasi district (West Java, Indonesia) were included after ethical approval from the Ethical Committee of the Faculty of Medicine, University of Indonesia. None of the subjects were under medication for systemic disease or under hormonal therapy. According to T-scores based on ultrasound densitometry of the calcaneous bone, 81 subjects were classified as normal (control group, mean T-

score -0.4, range -0.7…-0.1), 143 subjects with osteopenia (-0.7, -2.2…-1.3), and 61 subjects with osteoporosis (-3.1, -3.5…-2.8). The mean age (range) in the control group was 51 (48-55) years, in the osteopenia group 53 (50-56) years and in the osteoporosis group 60 (54-63) years.[22-25]

For PCR-RFLP analysis of OPG (TNFRSF11B) polymorphism at T950C (rs2073617), peripheral blood samples were used. For DNA isolation, 3 mL blood was inserted into test tubes with 9 mL of lysis solution (1.45 M NH_4Cl, 5 mM EDTA, 0.1 M anhydrous $KHCO_3$), incubated at room temperature for 10 min and centrifuged at 1500 rpm for 10 min. Supernatant was discarded to leave sediment of mononuclear leukocytes, and the process was repeated to obtain a white pellet and supernatant with no red blood cells. The pellet was dissolved in 2 mL of cell lysis solution (10 mM tris-HCl, pH 8, 0.25 mM EDTA, 0.5% SDS), incubated in a water bath at 37°C for 30-60 min, and after adding 1.3 mL of protein precipitation solution (5M ammonium acetate), centrifuged at 3000 rpm for 15 min at 4°C. The supernatant was decanted into a new tube with 2.3 mL of cold isopropanol, inverted 20-30 times until appearance of filament strands, and incubated at 20°C for 16-18 h. Centrifuging at 3000 rpm and 4°C for 5 min provided a white precipitate containing DNA. The supernatant was discarded and 1.3 mL of 70% ethanol was added, tube inverted to wash DNA, and centrifuged at 4°C and 3000 rpm for 5 min. Supernatant was discarded and DNA dried in open air for about 2 h. DNA was rehydrated with 200-300 mL TE solution (Tris-HCl EDTA) and incubated for 2 h at 37°C in a water bath. The solution was transferred into sterile 1.5 mL tubes to be stored at -20°C until further examination.

For PCR amplification, a Perkin Elmer GeneAmps PCR System 9700 was used with PCR Kit Bioline PCR mix with primers and 5 mL of DNA to a 25 mL total reagent volume. The primer sequences for amplification at rs2073617 were (forward) 5'-GGGGGATCCTTTCCGCCCCA-3' and (reverse) 5'-GTATCGCCTGCCTTTGATCAGT-3'. The amplification was done in 35 cycles with initial denaturation at 94°C for 5 min, then in cycles consisting of denaturation at 94°C for 30 s, annealing at 54°C for 30 s and elongation at 72°C for 30 s, and final extension at 72°C for 7 min. For verification, the resulting amplicons were subjected to electrophoresis on 3% agarose gel at 80 V for 30 min, illuminated with UV light and photographed. The target fragment was 147 bp in size. RFLP analysis was initiated by adding 0.5 mL of HincII restriction cutting enzyme (NEB) to 1 mL buffer solution, 0.5 mL BSA and 9 mL pure water to the DNA amplicon, incubating at 37°C for 4 h, and inactivating by incubation at 60°C for 20 min. The resulting DNA fragments were separated by electrophoresis on 3% agarose gel at 80 V for 1 h, illuminated with UV light and photographed. In case of the wild type (TT), no cutting of the 147 amplicon is seen, whereas the homozygous mutant genotype (CC) is indicated by two fragments (110 and 37 bp), and the heterozygous genotype (TC) by three fragments (147, 110 and 37 bp). DNA sequencing with the Sanger method was performed by 1stBase (Malaysia), to confirm that the amplified region and the fragments recognized by the restriction cutting enzyme are the desired targets. Sequencing included stages of PCR product purification, sequencing cycles, precipitation, sequencing back-cycle and analysis of the nucleotide base sequence.

Chi-square testing was mainly used for the frequency differences of the genotypes and alleles in the test groups, and for the genotype frequencies with respect to the Hardy-Weinberg equilibrium (HWE). To assess the association of genetic polymorphisms with risk of osteoporosis, odds ratios (OR) were also determined. Significance was generally assumed at p-values less than 0.05.

III. RESULTS

Examples of the PCR-RFLP results are shown in Figure 1, and of sequencing results in Figure 2. The results in terms of the distribution of genotypes and alleles with respect to the corresponding BMD status are shown in Table 1, and the related odds ratios in Table 2.

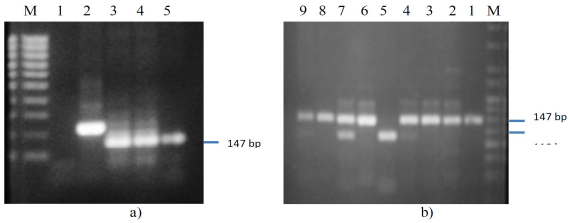


Figure 1. a) PCR results: M = 100 bp ladder; 1 = negative control; 2 = positive control (GADPH primer); 3 to 5 = 147 bp amplicon fragments; b) RFPL results: M = ladder of 25 bp; 1, 2, 3, 4, 8, 9 = wild-type (TT, no cutting); 5 = homozygous mutant (CC, 110 and 37 bp fragments, latter not visible); lane 7 = heterozygous genotype (TC, with 147, 110 and 37 bp fragments)

Table 1. Distribution of genotypes and alleles with respect to BMD status

	Genotype				Allele		
Group / type	TT	TC	CC	p	T	C	p
All N (%)	180 (63.2)	96 (33.7)	9 (3.2)	< 0.001	456 (80)	114 (20)	< 0.001
Normal N(%)	48 (26.7)	28 (29.2)	5 (55.6)	0.215	124 (27.2)	38 (33.3)	0.327
Osteopenia N(%)	89 (49.4)	52 (54.2)	2 (22.2)		230 (50.4)	56 (49.1)	
Osteoporosis N(%)	43 (23.9)	16 (16.7)	2 (22.2)		102 (22.4)	20 (17.5)	
Mean T-score	-1.73	-1.53	-1.39	0.330	-	-	

Table 2. Odds ratios (OR) of the genotypes and alleles with respect to BMD status

Chi-square test; * Fisher test

		Osteopenia+ Osteoporosis		Normal			
Group/type		N	%	N	%	p	OR (CI 95%)
Genotype	CC	4	44.4	5	55.6	0.120*	0.29 (0.08-1.13)
	TC	68	70.8	28	29.2	0.658#	0.88 (0.51-1.53)
	TT	132	73.3	48	26.7		Control
Allele	C	76	66.7	38	33.3	0.194#	0.75 (0.48-1.16)
	T	332	72.8	124	27.2		Control

Chi-square test; * Fisher test

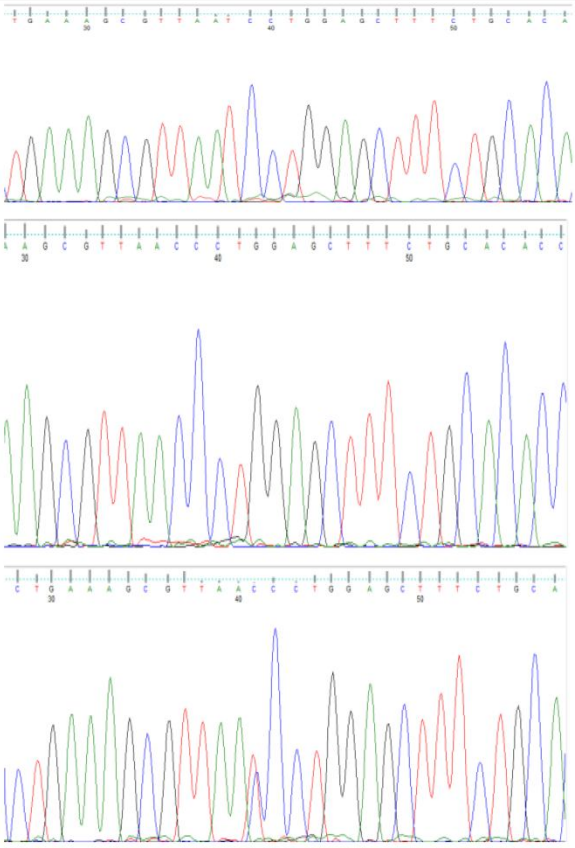


Figure 2. Sequencing results (from the top): wild-type (TT), homozygous mutant (CC), and heterozygous genotype (TC)

The observed genotype distribution was consistent with HWE for all subjects (χ^2 = 0.789, p = 0.374, N = 285), control group (χ^2 = 0.113, p = 0.737, N = 81), osteopenia group (χ^2 = 3.420, p = 0.064, N = 143) and osteoporosis group (χ^2 = 0.114, p = 0.736, N = 61).
The results summarized in Tables 1 and 2 indicate no significant association of the genotype (or allele) of the OPG T950C polymorphism with the BMD status or risk of osteoporosis in post-menopausal Indonesian women.

DISCUSSION

The results indicate no significant association of the T950C (rs2073617) polymorphism of the OPG (TNFRSF11B) gene with osteoporosis (BMD) in postmenopausal Indonesian women. No comparable previous results are known from the Indonesian population, but the results are in general agreement (Table 3) with those from most studies on the corresponding association elsewhere, although few early studies claim at least marginally significant association.[16,17,26] Differences have been reported between European and Asian populations in the association of OPG polymorphisms with BMD, and such association appears to be generally significant only for certain other OPG polymorphisms, such as G1181C (rs2073618), A163G (rs3102735) and C1217T (rs3102734).[10,26,30,32]

Table 3. Genotype distributions in OPG polymorphism (T950C) studies; F = female, M = male

Study	Origin (N, sex)	Normal (healthy) TT/TC/CC, %	Osteoporosis TT/TC/CC, %	Association[1)]
This work	Indonesian (285, F)	59.2/34.6/6.2	70.5/26.2/3.3	No
Arko et al.[11]	Slovenian (103, F)	-	25.2/53.4/21.4	No
Brändström et al.[13]	Swedish (858, F)	26.4/49.1/24.5 (BMD range)		No
Ueland et al.[14]	Australian (1468, F)	22.8/50.2/27.0 (BMD range)		No
Ohmori et al.[16]	Japanese (394, F)	43.4/42.6/14.0 (BMD range)		Yes
Langdahl et al.[17]	Danish (434, F)	24.4/47.5/28.1	21.4/54.4/24.2	Yes
Rhee et al.[15]	Korean (251, F)	33.1/50.0/16.9	-	Not tested
Korzon-Burakowska et al.[28]	Polish (95, M+F)	30.5/53.7/15.8	-	Not tested
Luo et al.[31]	Chinese (360, M+F)	33.3/43.3/23.3	-	Not tested

1) with BMD/osteoporosis

Postmenopausal osteoporosis involves loss of both bone mass and quality (microstructure and tissue composition), with degradation mechanisms that are not yet fully understood. Partly for this reason, the current drug treatments for post-menopausal osteoporosis do not eliminate bone fractures.[33] Although there is indicated association between OPG polymorphism and BMD, the association is mostly not particularly strong, even when used in combination.[18-26] Nevertheless, apart from a potential role as markers for likely postmenopausal osteoporosis, the polymorphisms can provide clues of the molecular level functions of OPG and the related regulatory network. These functions are not limited to those in bone metabolism, and while also the present work found no significant association of OPG T950C polymorphism and BMD, the same polymorphism has been reported to show significant association e.g. with coronary arterial disease.[26-31]
The known associations of BMD with environmental, nutritional, lifestyle and hormonal factors are generally stronger than those reported for OPG polymorphisms, and therefore tend to dominate the discussion on prevention and treatment of osteoporosis. However, the multifactorial nature of osteoporosis and the observed rather unique genotype distribution of OPG T950C polymorphism also imply that further work is of interest with a wider sampling from other parts of Indonesia.

In conclusion, the results showed that the frequencies of genotypes (TT, TC and CC) or alleles (T and C) of the OPG T950C (rs2073617) polymorphism were not significantly different in the three BMD groups (normal, osteopenia and osteoporosis, p > 0.05) of postmenopausal Indonesian women. Both for all subjects and for all three BMD groups separately, the genotype distributions were consistent with the Hardy-Weinberg equilibrium. In comparison to results from comparable studies elsewhere, however, the frequencies of the genotype CC (3.2% for all subjects) and allele C were low, 3.2% and 20.0% for all subjects, respectively.

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