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VDR gene polymorphism and trace elements in Thai postmenopausal women with risk of osteoporosis: Cross -sectional study

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ABSTRACT

Osteoporosis is the loss of bone density and it increases the risk of fracture. The *Fok*I polymorphism (C>T; rs2228570) of the vitamin D receptor (VDR) has a significant correlation with bone mineral density (BMD) reduction. Deficiencies of trace elements increase the risk of osteoporosis during menopause. This study aimed to compare and correlate bone-related parameters and trace elements and to identify *Fok*I genotypes and estimate its osteoporosis risk between normal and risk groups of postmenopausal women. Seventy subjects were randomly recruited in this study. BMD was determined by a quantitative ultrasound bone densitometer. The normal group ($N = 24$) included women with normal BMD status, while the low-BMD group ($N = 46$) included women with osteoporosis and osteopenia. Serum alkaline phosphatase (ALP), calcium (Ca), and magnesium (Mg) were measured by an automatic analyzer. Serum zinc (Zn) and selenium (Se) were extracted and analyzed by inductively coupled plasma-optical emission spectrometry. The *Fok*I genotypes of the VDR gene were amplified and identified by the polymerase chain reaction-restriction fragment length polymorphism. BMD was significantly negatively correlated with ALP and Ca ($r = -0.239$ and -0.673) and positively correlated with Mg and Zn ($r = 0.327$ and 0.383). The homozygous recessive (TT) genotype of the *Fok*I polymorphism was susceptible to osteoporosis (odds ratio = 2.69). We concluded that *FokI* Single nucleotide polymorphisms and bone markers may be useful in osteoporosis management in Thai postmenopausal women.

INTRODUCTION

Osteoporosis progresses without any symptoms until a bone fracture occurs. Osteoporotic fractures are a common problem in public health control and intervention, which relate to increasing immobility and mortality, affecting quality of life, and high economic impact due to extended hospital stay (Black and Rosen, 2016; Kawalkar, 2015). Intervention policies are necessary to diagnose and reduce the risk of fractures (Si *et al*., 2015; Williams *et al*., 2021). There are various associated factors, such as female gender (Black and Rosen, 2016; Pietschmann *et al*., 2009), old age (Aspray and Hill, 2019; Giusti and Bianchi, 2014), calcium intake (Cano *et al*., 2018), vitamin D level (Cano *et al*., 2018; Weaver *et al*., 2016), and genetic variations (Conti *et al*., 2015; He *et al*., 2015; Kubota *et al*., 2001). There are various genes involved in osteoclast proliferation, which are associated with bone mass reduction or osteoporotic fractures, including the vitamin D receptor (VDR), estrogen receptor, and collagen type I alpha-I genes (Stewart and Ralston, 2000).

Vitamin D and its active metabolites participate in the processes of bone tissue mineralization, maintaining calcium homeostasis, and bone remodeling, which are mediated through the VDR (Ahn *et al*., 2009). The variations VDR gene polymorphisms have been reported, and VDR genotypes are associated with bone diseases, including multiple sclerosis, osteoporosis, vitamin D-dependent rickets type II (Cantorna and Mahon, 2004; Jiang *et al*., 2020; Valdivielso and Fernandez, 2006), and essential hypertension (Banjabi *et al*., 2020). VDR

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gene polymorphisms occur in coding or noncoding regions, which can be changed in an amino acid sequence and also affect VDR expression (Uitterlinden *et al*., 2004). Single nucleotide polymorphisms (SNPs) of the VDR gene can be identified with the proper restriction endonucleases such as *FokI*, *ApaI*, *TaqI*, and *BsmI* by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique. Recently, VDR gene variants and the risk of osteoporosis have been associated with ethnic differences (Abbasi *et al*., 2012; Eisman, 1995; Jiang *et al*., 2020; Zintzaras *et al*., 2006). The *Fok*I polymorphism (C>T; rs2228570) of the VDR gene was significantly correlated with bone mineral density (BMD) reduction at the lumbar spine and elevation of hip bone loss in osteoporotic Mexican-American women (Gross *et al*., 1996). The association of the haplotype of *Taq*I, *Fok*I, and *Bsm*I SNPs of the VDR gene had been reported where the T allele was a risk factor for osteoporosis in an Indian population (Singh *et al*., 2013). *Fok*I SNPs could be a link between calcium and vitamin D intake, BMD level, and osteoporosis in obese Iranian women (Moradi *et al*., 2017). *Fok*I SNPs were also a significant risk factor for osteoporosis in Thai postmenopausal women with osteoporosis, and the TT genotype was susceptible to osteoporosis [odds ratio (OR) = 2.30] (Techapatiphandee *et al*., 2018).

Nutritional disturbances, especially deficiencies of trace elements and vitamins, pose increased risk for osteoporosis during menopause. Elements maintain skeleton development and function in the bone matrix (Howard *et al*., 1992). Abnormal metabolism of trace elements may play a role in osteoporosis development (Liu *et al*., 2009; Nieves, 2005; Odabasi *et al*., 2008). In addition, trace element supplementation with or without calcium (Ca) can increase BMD in postmenopausal women (Saltman and Strause, 1991). Early BMD screening for osteoporosis in Thai postmenopausal women is needed to focus on the community level (Sudjaroen and Thongmuang, 2019; Sudjaroen and Thongmuang, 2020), and calcium and vitamin D supplementation in a pilot study improved bone biochemical parameters (Sudjaroen and Thongmuang, 2018). This study aimed to compare and correlate bone-related parameters (i.e., BMD, Ca and alkaline phosphatase, and ALP) and trace elements [magnesium (Mg), zinc (Zn), and selenium (Se)] and to identify the genotypes of *Fok*I SNPs and estimate its osteoporosis risk between normal and risk groups of postmenopausal women.

MATERIALS AND METHODS

Subject recruitment

This research was a cross-sectional study during the period from December 2020 to March 2021. Approximately 1,500 participants were in the Public Health Service Program in the Osteoporosis Prevention Program, which was under the responsibility of the Samut Songkhram Provincial Government Office and Samut Songkhram Education Centre, Suan Sunandha Rajabhat University. Seventy subjects were randomly recruited in this study. Inclusion criteria were as follows: postmenopausal women who 1) were aged ≥ 55–65 years, 2) were conscious and interactive, and 3) had no serious symptom conditions. Exclusion criteria were as follows: women who had 1) complications with metabolic bone diseases, 2) a previous accident with a fracture, and 3) a pathological fracture (Gao *et al*., 2015). Anthropometric data were recorded by interviews during health service provision. The Ethics Committee of Suan Sunandha Rajabhat University approved this research protocol (COA.1-050-2020). The Director of the Osteoporosis Prevention Program permitted work on this research. All subjects were informed and consented.

BMD measurements

BMD was determined by a calcaneal quantitative ultrasound bone densitometer (SONOST-2000, OsteoSys, Korea), and the instrument operation and data interpretations were made according to manufacturer instruction. Interpretation was made according to BMD status, which was categorized as osteoporotic (T score at or below −2.5), osteopenic (T score between −1.0 and −2.5), and normal (T score at above −1.0) postmenopausal women. The normal postmenopausal women group (N = 24) had a normal BMD status, and the low-BMD postmenopausal women group (N = 46) included the osteoporotic and osteopenic status.

Blood collection and biochemical measurement

Each 5 ml blood sample was obtained by venipuncture from the median cubital vein during the morning (7–9 a.m.) and drawn into clotting blood and Ethylenediaminetetraacetic acid (EDTA) tubes for 3 and 2 ml, respectively. The clotting blood tube was further centrifuged, and serum was separated within 2 hours (Young and Bermes, 1999). Serum ALP, Ca, and Mg were measured by an automatic analyzer, COBAS c501 (Roche-Diagnostics, Rotkreuz, Switzerland). Zn and Se from the serum in the clotting blood tube were extracted and analyzed (Roychowdhury *et al*., 2002; Wach *et al*., 2018) by inductively coupled plasma-optical emission spectrometry (Avio200, Perkin Elmer, Thailand).

DNA extraction and genotype analysis

Whole blood contained in EDTA tubes was prepared for genomic DNA extraction by using the QIAamp Blood DNA Mini Kit (QIAGEN Thailand, Bangkok, Thailand), and genomic DNA was stored at −20°C. Genomic DNA was amplified along with the genotype of the *Fok*I (rs2228570) SNPs of the VDR gene by PCR-RFLP. The primers were reverse primer (3¢′-TTGTACCCTGCCCGCAAGAAA-5′¢) and forward primer (5′¢-ACCAAGGATGCCAGCTGG-3′¢). The PCR mixture used was OnePCR Ultra Supermix with a fluorescent dye (Bio-Helix, Gibthai, Thailand). The reaction conditions were optimized for initial denaturation at 94°C for 5 minutes followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds and extension at 72°C for 60 seconds, and one step of final extension at 72°C for 10 minutes in a thermal cycler (Prasad *et al*., 2021; Techapatiphandee *et al*., 2018). The amplified PCR product was 266 bp, which was confirmed by agarose gel electrophoresis. The PCR amplified product (20 ml) was conducted with RFLP by using 0.4 ml of the *Fok*I restriction endonuclease enzyme (R0109S, NEB), 2.5 ml of reaction buffer, and 2.1 ml of autoclaved water (total volume of reaction mixture = 25 ml). The reaction mixture was incubated at 37°C for 60 minutes in a water bath for making DNA fragments (Prasad *et al*., 2021) and genotyped in each sample by DNA fragment separation on 2.5% agarose gel electrophoresis along with DNA ladder (VC 100 bp DNA Ladder, Vivantis Technologies, Malaysia). DNA electropherogram was

performed by Bio-Rad Universal Hood II-GelDoc System (Bio-Rad Laboratories-Segrate, Italy). *Fok*I SNPs were 266 bp (uncut) for the homozygous dominant (CC) genotype; 184 and 63 bp (cut) for the TT genotype; and 266, 184, and 63 bp (cut/uncut) for the heterozygous (CT) genotype (Techapatiphandee *et al*., 2018). Direct sequencing confirmed genotypes in 15% of randomized samples.

Statistical analysis

Descriptive data was represented by using mean, standard deviation, and frequency. The independent *t*-test was used for the comparison of individual and biochemical parameters between the normal and risk (low-BMD) groups. Pearson's correlation was used to analyze the relationship between individual and biochemical parameters. Chi-square was used to calculate odd risk estimation on different *Fok*I genotypes. Statistical analysis was conducted by the SPSS 21.0 software.

RESULTS AND DISCUSSION

Bone-related parameters and trace elements in postmenopausal women

Age between the normal and low-BMD (risk) groups was not significantly different. ALP and serum Ca of the risk group were significantly higher than in the normal group ($p = 0.035$ and < 0.0001); therefore, they were within reference ranges. Serum Mg, Zn, and Se of the risk group were significantly lower than in the normal group ($p = 0.012$, 0.04, and 0.028, respectively). Serum Zn in the risk group and Se in both groups were lower than reference ranges (Table 1). BMD was significantly negatively correlated with ALP and Ca ($r = -0.239$ and -0.673) and positively correlated with Mg and Zn ($r = 0.327$ and 0.383). Serum calcium was significantly negatively correlated with all trace elements. The correlation of serum Zn and Se was the strongest ($r = 0.824$) due to cofactors of antioxidant enzymes (Table 2).

Previous studies reported higher Ca and ALP (within reference ranges) in women with osteoporosis (Ali, 2018; Sasmita *et al*., 2015). Serum Ca was controlled and maintained homeostasis between the serum and bone compartment. Increasing of serum Ca and BMD reduction in osteoporotic women were implied to have a negative correlation between serum Ca and BMD status (Ali, 2018; Rana, 2013). ALP is a clinical marker of bone metabolism, and its activity arises from the bone and liver. The ALP level of the risk and normal groups was significantly different; therefore, it was still within the reference range. A significant finding was represented in the relationship of the BMD status or osteoporosis and ALP (Narula *et al*., 2013). Mg is an important mineral for bone cell function especially osteoblast mitogenicity. Thus, when Mg has a deficit, the growth of osteoblasts will be inhibited (Rude and Gruber, 2004). A significant difference of Mg between the osteoporotic and healthy postmenopausal women was also observed in red blood cells, which was described by the Mg transport mechanism into the cell in osteoporosis (Odabasi *et al*., 2008). Zn is an essential element for over 200 enzymes, collagen production, and bone mineralization (Hyun *et al*., 2004). Se is also important for human health, and depletion of Se will affect bone tissue (Chariot and Bignani, 2003). Oxidative stress is implicated in postmenopausal osteoporosis by loss of balance between antioxidative and oxidative markers. Monitoring of oxidative stress-related markers is useful for the diagnosis and prognosis of osteoporosis (Zhao *et al*., 2021). Hence, reduction of serum Mg, Zn, and Se in osteoporosis may affect the antioxidant status, especially antioxidant enzymes. Our finding was summarized in that serum Ca and ALP in the risk group were higher than in normal postmenopausal women, and serum Mg, Zn, and Se will reduce along with BMD during osteoporosis progress. High serum Ca in postmenopausal women is a risk for bone loss, and estrogen deficit increases bone turnover and bone resorption. Hence, the hormonal and BMD status in females are important factors, which

Table 1. Comparison of normal and low BMD groups among postmenopausal women.

Group	Frequency (%)	Age (years)	BMD (T-score)	ALP (U/l)	Ca (mg/dl)	Mg (mg/dl)	Zn (mg/dl)	Se (mg/dl)
Normal BMD [a]	24 (34.3)	59.6 ± 7.45	0.69 ± 0.87	77.4 ± 15.43	9.1 ± 0.40	2.03 ± 0.14	61.4 ± 19.9	2.96 ± 1.16
Low BMD [b]	46 (65.7)	61.2 ± 10.5	−1.98 ± 0.86	87.0 ± 20.90	9.9 ± 0.71	1.87 ± 0.19	46.4 ± 17.9	2.28 ± 1.22
p-value	-	0.081	-	0.035*	<0.0001**	0.012*	0.04*	0.028*
Reference range	-	-	> −1.0	30–120	8.2–10.2	1.8–2.5	50–170	8–25

[a] Normal BMD was defined as postmenopausal women who had T-Score were above −1.0.

[b] Low BMD was included osteopenia and osteoporosis women; and T-Score were within −1.0 to −2.5 and lower than −2.5, respectively.

*Statistically significant at $p < 0.05$; ** Statistically significant at $p < 0.001$.

Table 2. Pearson's correlation coefficient (*r*) between BMD, age and biochemical parameters in postmenopausal women.

	BMD	ALP	Ca	Mg	Zn	Se
BMD	1	−0.239*	−0.673**	0.327**	0.383**	0.221
ALP	−0.239*	1	0.172	−0.01	−0.25	0.33
Ca	−0.673**	0.172	1	−0.267*	−0.449**	−0.374**
Mg	0.327**	−0.01	−0.267*	1	0.172	−0.041
Zn	0.383**	−0.25	−0.449**	0.172	1	0.824**
Se	0.221	0.33	−0.374**	−0.041	0.824**	1

*Statistically significant at $p < 0.05$ level (two-tailed); ** Statistically significant at $p < 0.01$.

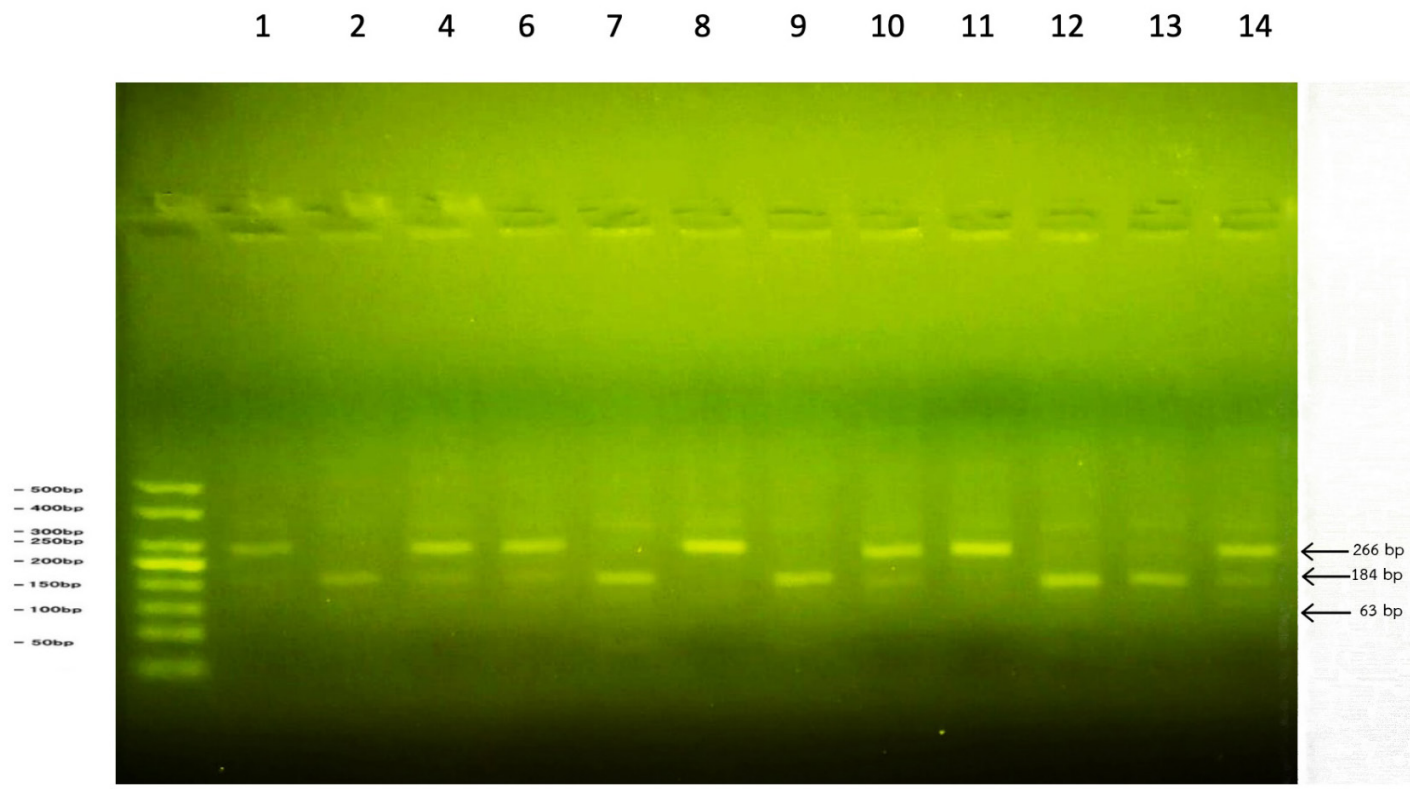

Figure 1. Identification of *Fok*I polymorphism on VDR gene by PCR-RFLP: (a) CC genotype was represented as 266 bp fragment (uncut) in Lanes 8 and 11; (b) CT genotype was represented as 266, 184, and 63 bp fragments (heterozygous) in Lanes 4, 6, 10, and 14; and (c) TT genotype was represented as 184 and 63 bp fragments (homozygous recessive) in Lanes 2, 7, 9, 12, and 13.

Table 3. OR estimation in *Fok* I polymorphism of VDR gene (codominant model) between normal and risk (low BMD) groups.

Genotype	Normal group (*n* = 24)	Risk group (*n* = 46)	OR (95% CI)	*p*-value
CC	9 (37.5%)	6 (13.0%)	0.45 (0.088–2.329)	< 0.001
CT	13 (54.2%)	32 (69.6%)	1.00 (0.359–2.789)	
TT	2 (8.3%)	8 (17.4%)	2.69 (0.508–4.882)	

should be addressed in osteoporosis studies (Dalemo *et al*., 2018; Lerner, 2006; Liu *et al*., 2019).

*Fok*I genotypes of VDR gene polymorphisms in postmenopausal women

The DNA electrophoresis of the *Fok*I genotypes identified a homozygous dominant (CC) for the 266 bp fragment, heterozygous (CT) for the 266, 184, and 63 bp fragments, and homozygous recessive (TT) for the 184 and 63 bp fragments (Fig. 1). The distribution of the T allele is more frequent in the risk group, and the TT genotype was high risk (OR = 2.69, 0.508–4.882) and susceptible to osteoporosis (Table 3). Our significant finding corresponded to a previous study, where it was reported that the *Fok*I genotypes of the VDR gene were related to osteoporosis and the TT genotype was high risk and most susceptible in Thai osteoporotic patients (Techapatiphandee *et al*., 2018). We confirmed that these SNPs were useful in osteopenia and osteoporotic postmenopausal women with asymptomatic appearances and no history of fracture for at least the past 3 years. There was supportive evidence for osteoporosis occurrence in tropical countries, which have enough sunlight for vitamin D production. The *Fok*I genotypes of the VDR gene were also related to primary hypertension (Prasad *et al*., 2021), periodontitis (Liu *et al*., 2020), and tumorigenesis (Rai *et al*., 2017). Identification of the *Fok*I SNPs was used to predict osteoporotic progression in postmenopausal women without symptoms or fractures.

CONCLUSION

We concluded that serum Ca and ALP were negatively correlated with BMD. However, serum Mg, Zn, and Se were positively correlated with BMD in postmenopausal women. It was implied that osteoporosis was related to antioxidant reduction by trace element depletion. Identification of the *Fok*I SNPs was used to predict osteoporotic progression in postmenopausal women without symptoms or fractures.

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AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

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CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVALS

The Ethics Committee of Suan Sunandha Rajabhat University approved this research protocol (COA.1-050-2020). The Director of the Osteoporosis Prevention Program permitted work on this research. All subjects were informed and consented.

DATA AVAILABILITY

The study data is available with authors.

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