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 FokI 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 FokI 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 = 46) included women with normal BMD status, while the low-BMD group (N = 24) 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 FokI 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 FokI 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 FokI polymorphism (C>T; rs228570) 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 TaqI, FokI, and BsmI 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). FokI SNPs could be a link between calcium and vitamin D intake, BMD level, and osteoporosis in obese Iranian women (Moradi et al., 2017). FokI SNPs were also a significant risk factor for osteoporosis in Thai postmenopausal women with osteoporosis, and the TT genotype was susceptible to osteoporosis \( \text{[odds ratio (OR) = 2.30]} \) (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)] to identify the genotypes of FokI 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 Songkram Provincial Government Office and Samut Songkram 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 \( \geq 55–65 \) 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 QIAGen Blood DNA Mini Kit (QIAGEN Thailand, Bangkok, Thailand), and genomic DNA was stored at \(-20^\circ\text{C}\). Genomic DNA was amplified along with the genotype of the FokI (rs228570) SNPs of the VDR gene by PCR-RFLP. The primers were reverse primer \((3\text{`-TTGTACCTCGCCGCAAAGAGA-5`})\) and forward primer \((5\text{`-ACCAAGGTGCCAGCTGG-3`})\). 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 \(94^\circ\text{C}\) for 5 minutes followed by 40 cycles of denaturation at \(94^\circ\text{C}\) for 30 seconds, annealing at \(58^\circ\text{C}\) for 30 seconds and extension at \(72^\circ\text{C}\) for 60 seconds, and one step of final extension at \(72^\circ\text{C}\) 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 FokI restriction endonuclease enzyme (R0109S, NEB), 2.5 ml of reaction buffer, and 2.1 ml of autoclaved water (total volume of reaction mixture \(= 25\text{ ml}\)). The reaction mixture was incubated at \(37^\circ\text{C}\) 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). FokI 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 FokI 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

| Group   | Frequency (%) | Age (years) | BMD (T-score) | ALP (U/l) | Ca (mg/dl) | Mg (mg/dl) | Zn (mg/dl) | Se (mg/dl) |
|---------|---------------|-------------|---------------|-----------|------------|------------|------------|------------|
| Normal BMD * | 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       |

*Normal BMD was defined as postmenopausal women who had T-Score were above −1.0.
*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 | −0.267* | −0.449** |
| 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 $p < 0.01$. 

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

**Table 2.** Pearson’s correlation coefficient ($r$) between BMD, age and biochemical parameters in postmenopausal women.
should be addressed in osteoporosis studies (Dalemo et al., 2018; Lerner, 2006; Liu et al., 2019).

FokI genotypes of VDR gene polymorphisms in postmenopausal women

The DNA electrophoresis of the FokI 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 FokI 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 FokI 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 FokI 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 FokI SNPs was used to predict osteoporotic progression in postmenopausal women without symptoms or fractures.

Table 3. OR estimation in FokI 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)   |         |

Figure 1. Identification of FokI 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.
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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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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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