EFFECT OF *GGCX* GENE POLYMORPHISM ON THE RESPONSES OF SERUM UNDERCARBOXYLATED OSTEOCALCIN AND BONE TURNOVER MARKERS AFTER TREATMENT WITH VITAMIN K2 (MENATETRENONE) AMONG THAI POSTMENOPAUSAL WOMEN IN PHRAMONGKUTKLAO HOSPITAL

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A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Research for Health Development
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ผลของความแตกต่างทางพันธุกรรมของยีน *GGCX* กับการตอบสนองของค่า undercarboxylated osteocalcin และดัชนีชีวเคมีการผลัดเปลี่ยนกระดูกในซีรั่ม ภายหลังการได้รับยาวิตามินเคสองในสตรีไทยวัยหมดระดู ในโรงพยาบาลพระมงกุฎเกล้า

นาย ทวี ทรงพัฒนาศิลป์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตร
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Thesis Title	Effect of *GGCX* gene polymorphism on the response of serum undercarboxylated osteocalcin and bone turnover markers after treatment with Vitamin K2 (Menatetrenone) among Thai postmenopausal women in Pramongkutklao Hospital
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ทวี ทรงพัฒนาศิลป์: ผลของความแตกต่างทางพันธุกรรมของยีน *GGCX* กับการตอบสนอง ของค่า undercarboxylated osteocalcin และ ดัชนีชีวเคมีการผลัดเปลี่ยนกระดูกในซีรั่ม ภายหลังการได้รับยาวิตามินเคสอง ในสตรีไทยวัยหมดระดู ในโรงพยาบาลพระมงกุฎเกล้า. (EFFECT OF *GGCX* GENE POLYMORPHISM ON THE RESPONSES OF SERUM UNDERCARBOXYLATED OSTEOCALCIN AND BONE TURNOVER MARKERS AFTER TREATMENT WITH VITAMIN K2 (MENATETRENONE) AMONG THAI POSTMENOPAUSAL WOMEN IN PHRAMONGKUTKLAO HOSPITAL) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : ศ.นพ.นิมิต เตชไกรชนะ,อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : ศ.นพ. บุญส่ง องค์พิพัฒนกุล 45 หน้า.

วัตถุประสงค์: เพื่อประเมินการตอบสนองของค่า undercarboxylated osteocalcin (ucOC) และ ดัชนีชีวเคมีการผลัดเปลี่ยนกระดูกในซีรั่ม ภายหลังการได้รับยาวิตามินเคสองในสตรีไทยวัยหมดระดู ที่มีความแตกต่างทางพันธุกรรมของยีน *GGCX*

รูปแบบการทดลอง: การศึกษาเภสัชพันธุศาสตร์ทางคลินิก

วิธีการศึกษา: ผู้หญิงวัยหมดระดูจำนวน 140 ราย ได้เข้าร่วมในการศึกษานี้ โดยจะได้รับการเจาะเลือดเพื่อตรวจหาระดับ ซีรั่ม ucOC ดัชนีชีวเคมีการผลัดเปลี่ยนกระดูก และ *GGCX* genotyping ทุกรายจะได้รับ menatetrenone 45 mg/d ร่วมกับแคลเซียมและวิตามินดี หลังจากนั้นจะมีการประเมินผลการตอบสนอง โดยประเมินการลดลงจากก่อนให้ยาของระดับซีรั่ม ucOC ที่ 3 เดือน ดัชนีชีวเคมีการผลัดเปลี่ยนกระดูก ที่ 1 และ 3 เดือน โดยเปรียบเทียบกันระหว่างกลุ่มที่มี GG genotype กับกลุ่มที่มี GA หรือ AA genotype

ผลการศึกษา: พบการลดลงอย่างมีนัยสำคัญของระดับซีรั่ม ucOC และ ดัชนีชีวเคมีการผลัดเปลี่ยนกระดูก เมื่อเทียบกับค่าเริ่มต้นก่อนให้ยาของทั้งกลุ่มผู้เข้าร่วมวิจัย และในกลุ่มที่มี GG genotype และกลุ่มที่มี GA หรือ AA genotype ($p<0.001$). แต่เมื่อเปรียบเทียบกันระหว่างกลุ่มที่มี GG genotype กับ กลุ่มที่มี GA หรือ AA genotype ไม่พบความแตกต่างอย่างมีนัยสำคัญ ($p >0.05$). ในการศึกษานี้ไม่พบอาการไม่พึงประสงค์ที่รุนแรงใดๆ.

สรุป: การใช้ Menatetrenone ร่วมกับแคลเซียมและวิตามินดีสามารถลด ระดับซีรั่ม ucOC และ ดัชนีชีวเคมีการผลัดเปลี่ยนกระดูกได้ ซึ่งเป็นกลไกเกี่ยวข้องกับการคงสภาพที่ดีของคุณภาพกระดูก อย่างไรก็ตาม ความแตกต่างทางพันธุกรรมของยีน *GGCX* ไม่มีผลในการลดลงของค่าต่างๆเหล่านี้

สาขาวิชา: วิจัยเพื่อการพัฒนาสุขภาพ ลายมือชื่อนิสิต.....

ปีการศึกษา: 2552 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

4989666720 : MAJOR RESEARCH FOR HEALTH DEVELOPMENT

KEYWORDS : menatetrenone / vitamin K2 / undercarboxylated osteocalcin / bone turnover markers/ GGCX gene

THAWEE SONGPATANASILP: EFFECT OF *GGCX* GENE POLYMORPHISM ON THE RESPONSES OF SERUM UNDERCARBOXYLATED OSTEOCALCIN AND BONE TURNOVER MARKERS AFTER TREATMENT WITH VITAMIN K2 (MENATETRENONE) AMONG THAI POSTMENOPAUSAL WOMEN IN PHRAMONGKUTKLAO HOSPITAL THESIS ADVISOR : PROFESSOR NIMIT TAECHAKRAICHANA M.D., THESIS CO-ADVISOR : PROFESSOR BOONSONG ONGPHIPHADHANAKUL M.D., 45 pp.

Objective: To evaluate the different response of serum undercarboxylated osteocalcin (ucOC) and bone turnover markers at three months after treatment with menatetrenone in Thai postmenopausal women, among differences in *GGCX* gene polymorphisms.

Design: Prospective clinical pharmacogenetic study

Method: One hundred and forty Thai postmenopausal women were enrolled to evaluate the response to 45 mg/d treatment with menatetrenone concurrently with calcium and vitamin D for 3 months. Baseline serum bone turnover markers and undercarboxylated osteocalcin (ucOC) levels were collected from all participants as well as demographic characteristics and GGCX genotyping. We evaluate the reduction from baseline of ucOC at 3 months and reduction from baseline of beta-CTx and P1NP at 1 and 3 months. We compared all these parameters between different groups of GGCX genotypes (GG-group and GA+AA-group) as the main interest.

Results: There are significant reduction of serum ucOC, beta-CTx and P1NP from baseline at 3 months by pair t-test and one-way repeated ANOVA analysis ($p<0.001$). in both GG- and GA+AA-group. But there are no significant difference when compare between genotypes ($p >0.05$). There were no-serious adverse events in this study.

Conclusion: Menatetrenone can effectively reduced serum ucOC as well as reduced beta-CTX and P1NP, which are the main mechanism to maintain the bone quality. *GGCX* polymorphism does not influence these reductions.

Field of Study: Research for Health Development Student's Signature .....

Academic Year: 2009 Advisor's Signature .....

Co-Advisor's Signature .....

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CHAPTER 1

Introduction

Rational and Background

Osteoporosis is characterized by the decreasing of bone strength[1] and is a major problem for the elderly population because it leads to an increase risk of fractures which subsequence impairs their quality of life [2,3] and increases mortality[4,5]. Therefore, fracture prevention is a primary aim of treatment for osteoporosis and also the main outcome for the study that evaluate the efficacy of drugs for treatment of this disease. The pharmacological agents used in treatment of osteoporosis are now can be classified into three categories. The first class of these drugs is the agents which decrease the bone remodeling by inhibit osteoclast activity. The use of these anti-catabolic agents in the treatment of osteoporosis such as bisphosphonates and selective estrogen receptor modulators (SERM) have led to a reduction in the occurrence of vertebral fractures and long bone fractures [6–8]. A second class of agents which used to treat osteoporosis called anabolic drugs. Its action is increasing bone mass and bone strength by the promotion of bone remodeling and new bone formation. Parathyroid hormone is a typical agent of this class. It increases bone formation more than it increases bone resorption, so, it lead to the improvement of bone mass and bone architecture along with a reduction of the incidence of fractures[9]. The last class of the anti-osteoporosis agent is called the agents with mixed actions. These agents will increase bone formation while also inhibit bone resorption at the same time[10]. These agents are such as strontium ranelate and a form of vitamin K2 called “menatetrenone”. Anyway, menatetrenone was considered to be the agent which demonstrates promotion of osteoblastogenesis and inhibition of osteoclastogenesis by several in vitro and in vivo studies[11-13]. Although, there are many clinical studies about effects of menatetrenone on bone formation and bone resorption, there results are still controversy. To explore the mechanism of actions of all these anti-osteoporosis agents in clinical situation, the surrogate outcomes such as bone mineral density (BMD) and biochemical marker of bone turnover (bone turnover markers, BTMs) are accepted. Especially, bone turnover markers, they can used to classify the mechanism of action of each drug into the anti-

resorptive agent by observing the reduction of bone resorption marker or define it to the bone-anabolic agent by observing the increasing of bone formation marker.

In the past, we used to understand that vitamin K is only necessary for blood coagulation. Now, the scientist found the new knowledge about vitamin K that besides its role for blood clotting, vitamin K is very important for the bone metabolism and may related to some important function of many proteins in other human tissues. There is a very important group of proteins in our body which can function properly depend on the adequacy of vitamin K. We call this group of protein as “vitamin K dependent proteins” (VKD protein)[14]. These proteins need posttranslational modification of amino acid in some positions which call “glutamate residues” (Glu) and change them into the gamma-carboxyglutamic acid (Gla), then, these proteins can work in the optimum function. The process of changing glutamate residues to gamma-carboxyglutamic residual or from Glu to Gla need vitamin K as a co-factor[15]. In case of vitamin K insufficiency, the glutamate residues of these proteins cannot be changed or incompletely changed into the fully-carboxylated form which may impair their normal biological activity. We also named these vitamin K dependent proteins as “Gla-containing proteins” due to the necessity for the full function of these proteins depended on the present of completeness of Gla-residual[15]. The vitamin K dependent proteins (VKD) which have not completed gamma-carboxylation will not work or cannot work properly, and we name them as “undercarboxyted form”(uc-form)[15]. The VKD proteins which were completely gamma-carboxylated and completely work their duties we name them as full-carboxylated form or mature form[15]. The Gla residues in these proteins have a strong ability to bind with Ca^{2+}(calcium binding ability [16]. At least, three vitamin K-dependent proteins have been identified in bones [16]. The most abundant non-collagenous protein of bone, osteocalcin (OC), is vitamin K dependent. It has also been called bone gamma-carboxyglutamic acid (Gla) protein (BGP). It is a small Ca^{2+}-binding protein that contains three Gla residues and it is indigenous to the organic matrix of bone, dentin, and possibly other mineralized tissues. Although the precise role of OC in bone metabolism is not fully understood, a number of findings suggest its importance in regulating bone mineralization, maturation, and remodeling[17,18]. Many of epidemiological studies have shown a relationship between vitamin K intake and bone health. The two largest of these

studies are the Framingham Heart Study and the Nurse's Health Study[19-21]. Both of these studies have indicated that vitamin K intake, measured by food frequency questionnaire, correlates with hip fracture risk and bone mineral density (BMD)[20]. In the Nurses' Health study[21] comprising 72,327 women aged 38–63 years, it shown that an intake of <109 mg/day of vitamin K was associated with a 30% higher risk of hip fractures than with higher vitamin K intakes. Hodges et al [22,23], reports of significantly reduced blood levels of vitamin K1, MK-7, and MK-8 in women and men with hip or vertebral fractures compared with controls. Several studies[24,,25] in Japanese also reported that reduced serum vitamin K1 or MK-7 is associated with reduced lumbar spine BMD in Japanese men and postmenopausal women.

Direct measurement of serum vitamin K level is very complicated. Nowadays, there is a development of the measurement of vitamin K status by using the serum level of undercarboxylated osteocalcin (ucOC) as a marker, which can be an indicator of deficiency or insufficiency of vitamin K in human body. The high ucOC level point that there is poor carboxylation status of OC and reflex the vitamin K insufficiency state. The carboxylation status of OC is also an independent risk factor for fractures. This fraction of ucOC is significantly increased in postmenopausal women, especially in elderly women[26]. Its serum levels return to normal values for young adults after treated with low doses of vitamin K1[27]. We also showed that ucOC negatively correlated with hip bone mineral density (BMD)[28] and that the fraction of ucOC is a powerful predictor of hip fracture [29,30]. However, there is no consensus about the cutoff level of ucOC level in serum for the vitamin K insufficiency. We believed that the level of ucOC in serum may different in each race. In Japan by Shiraki et al [31], and most of expert in this field, agree to use the ucOC level at 4.0 ng/ml as a cutoff point. Bunyaratavej et al[32], studied the level of ucOC in Thai premenopausal women in 357 case and found that the average of serum ucOC was 2.69 ng/ml (95% CI, 2.48-2.90), so he conclude that the level of ucOC in serum at 3.0 ng/ml is the cutoff level for Thai women. In contrast, Plantalech L, et al[26], had study ucOC level in Caucasian, he conclude that the cutoff level should be 1.65 ng/ml. The dissimilarity between these results might be come from the difference in method used for measurement of ucOC, difference in dietary habit, and the last may be due to the racial difference in genetic basis. There are few clinical studies for vitamin K

which have fracture risk reduction as primary outcome of the study. Most of them done in Japan and used menatetrenone (MK-4) as the study agent. A study by Shiraki et al,[33] which is the landmark study in postmenopausal women has been done in the year 2000. This is the randomized controlled trial (RCT) of 241 postmenopausal women, average age of 67.2 years old and 2 years follow up. He found that menatetrenone can significantly decrease vertebral fracture (p=0.02) about 53%, but cannot reduce the non-vertebral fracture risk.

However, the results about treatment of vitamin K in postmenopausal are still controversy. As mentioned above, undercarboxylated osteocalcin (ucOC) was clearly negative correlated with BMD and that administration of vitamin K decreased ucOC. Vitamin K may have some effect on BTMs as well as preservation of BMD. These variation in efficacy of vitamin K may be due to the type of vitamin K administration (K1 or K2), the difference of absorption and transportation in our body which depend on lipoproteins, the tissue specific differences in uptake of vitamin K (K2 is better than K1 in extra-hepatic tissue)[18] and the last reason may be due to differences in efficiency of enzyme GGCX which have genetic polymorphism. Among *GGCX* gene polymorphism, only one single nucleotide polymorphism (SNP) reported, found to correlate with BMD[34]. Report by Kinoshita et al [34] in 2007, shown that the *GGCX* gene is on chromosome 2p12 and the mutation in exon 8 in the position *rs699664* (Arg325Gln) is correlated with BMD.

Research Objectives

1. To evaluate the different effects among the level of serum ucOC response at three months after treatment with menatetrenone in Thai postmenopausal women, who have differences in *GGCX (rs699664, A/G)* gene polymorphisms, between the patients which have AA or GA genotypes and the patients which have GG genotype.
2. To find the pattern of bone turnover markers (BTMs) response in Thai postmenopausal women treated with menatetrenone, among differences in *GGCX (rs699664, A/G)* gene polymorphisms.

3. To find the difference of baseline ucOC and bone turnover markers among the differences in *GGCX (rs699664, A/G)* gene polymorphisms.
4. To find the distribution frequency of *GGCX (rs699664, A/G) gene* polymorphisms. (AA, GA and GG genotype) in Thai postmenopausal women

Limitations

There is some limitation in find out these eligible subjects for this study in one-investigational center. Multi-center studies for this protocol may be needed. To study the gene polymorphism, we need a specific laboratory which have a facility to do all these genotyping processes. Faculty of Medicine, Ramathibodi Hospital is one of the leader hospitals that capable to perform this polymorphism study, we need to be incorporate with.

Operational Definitions

Vitamin K: Vitamin K is a family of structurally similar, fat-soluble vitamin, 2-methyl-1,4-naphthoquinones, including phylloquinone (K1), menaquinones (K2). The structural difference is in their side chain)[35] (figure 1.1). Vitamin K1 possesses a phytyl group (partially saturated polyisoprenoid group), while K2 possesses a repeating, unsaturated trans-polyisoprenyl group. Phylloquinone is found in higher plants and algae, with the highest concentrations found in green leafy vegetables[35]. The most common form of vitamin K2 in animals is menaquinone-4 (menatetrenone; MK-4), produced by the processing of exogenous and bacterial naphthoquinones[35].

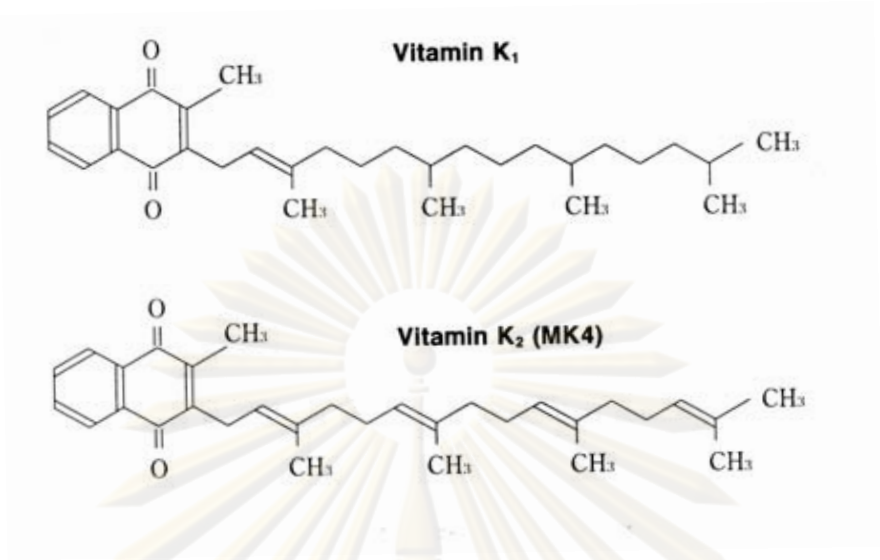


Fig 1.1 Structure of vitamin K

Vitamin K dependent proteins: Vitamin K is a cofactor in a number of biochemical pathways. Those most commonly associated with vitamin K are the vitamin K-dependent carboxylation reactions, and the proteins needed to be under this process for fully biological function are call “vitamin K dependent proteins” (VKD). There are many VKD proteins in our body, in the hepatic tissue are prothrombin (factor II), factor VII, IX and X, protein S, protein C and protein Z. For the extra-hepatic tissues, such as bone, there are osteocalcin (OC), matrix-Gla protein (MGP), protein S.

Osteocalcin (OC): is exclusively synthesized by osteoblasts and odontoblasts. Human osteocalcin having 49 amino acids. Its amino acid sequence (primary structure) has been highly conserved during evolution, and it is the most abundant non-collagenous protein in the bones and dentine of vertebrates. There are 3 glutamate (Glu) residues at positions 17, 21, and 24 that can be posttranslationally modified to gamma-carboxyglutamic acid (Gla residues)[18]. Although the precise role of OC in bone metabolism is not fully understood, a number of findings suggest its importance in regulating bone mineralization, maturation, and bone remodeling.

Undercarboxylated Osteocalcin (ucOC): is the fraction of the osteocalcin in the circulation which is not fully carboxylated. In healthy adults, a very small portion of blood

clotting factors is undercarboxylated. In contrast, a substantial portion of circulating OC is undercarboxylated. Thus, circulating undercarboxylated OC (ucOC) is a more sensitive measurement of vitamin K status than are the conventional blood coagulation tests[18]

Bone Turnover Markers (BTMs): Bone is a metabolically active tissue that undergoes continuous remodeling by two counter acting processes, named bone formation and bone resorption. These processes rely on the activity of osteoclasts (resorption), osteoblasts (formation). Under normal conditions, bone resorption and formation are tightly coupled to each other. Bone turnover markers or some time we call the "biochemical markers of bone turnover" are the biochemical molecules which are products of the remodeling process and reflex the activity of both cells, osteoclasts (bone resorption markers) and osteoblasts (bone formation markers). We can measure these biochemical molecules in serum and they will tell us about the status of bone resorption process and bone formation process of that subject.

Bone resorption markers: the majority of bone resorption markers are degradation products of bone collagen from the process of osteoclastic bone resorption. They reflex the activity of the osteoclast in remodeling process. The most useful bone resorption markers in clinical practice are beta-CTx (C-terminal telopeptide) and NTx (N-terminal telopeptide). These also the urinary makers, such as urinary PYD (urinary pyridinoline) and urinary DPD (urinary pyridinoline) which were used in some institutes.

Bone formation markers: are products of active osteoblasts expressed during different phases of osteoblasts development, and also products of bone matrix synthesis by those osteoblasts in bone formation process. The most useful bone formation markers in clinical practice are BSALP (bone specific alkaline phosphatase), P1NP (type-1 collagen N-terminal propeptide), P1CP (type-1 collagen C-terminal propeptide) and NMID-OC (N-terminal-MID osteocalcin).

Gamma Glutamyl Carboxylase (GGCX): is an essential enzyme that converts glutamate (Glu) residues in the VKD proteins into gamma-carboxyglutamic acid (Gla) residues. It is the vitamin K dependent enzyme which needs vitamin K as a co-factor. When vitamin K going into our body, it has to change into "hydroquinone" form and then

together with this enzyme (GGCX) it modify the Glu residuals into Gla residuals)[18].(figure 1.2)

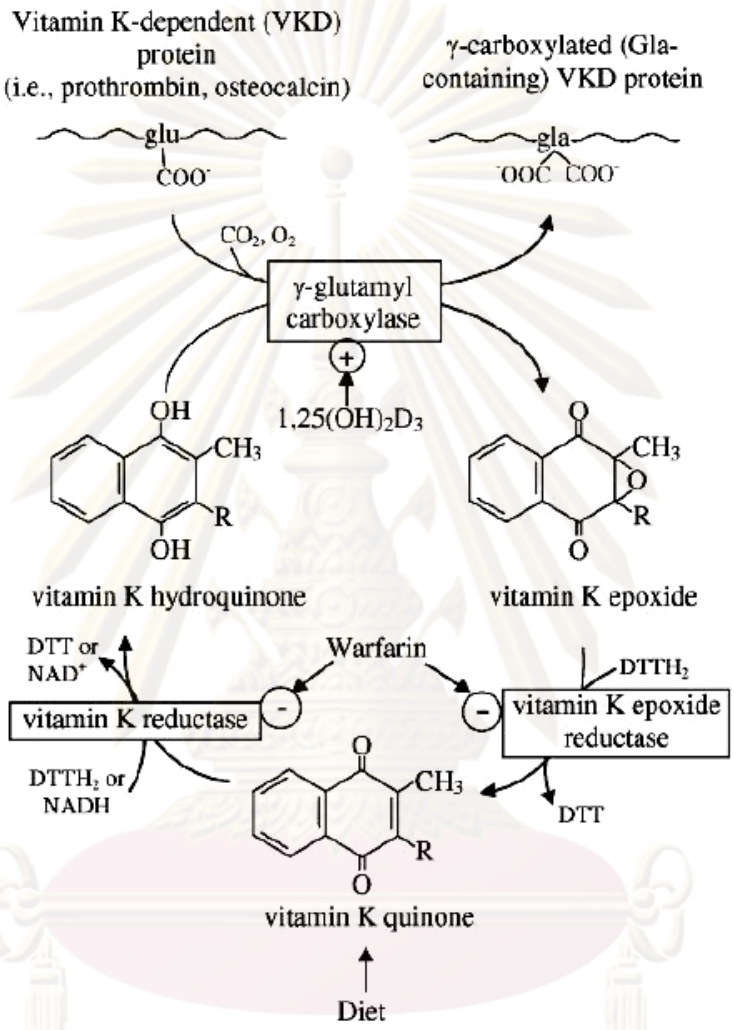


Fig 1.2 Vitamin K cycle and γ-glutamyl carboxylase enzyme

Single nucleotide polymorphism (SNP): variation in a single nucleotide on DNA sequences, which encode for a protein. We study this variation because of protein that encoded from this gene will have some differences in their functions, we call these "polymorphism".

Gamma Glutamyl Carboxylase Genes: is the gene encoded for enzyme gamma-glutamyl carboxylase. The genomic structure of *GGCX gene* was elucidated in 1997[36]. It is reported that the rare mutations of *GGCX gene* with amino acid substitution (Leu395Arg, Trp501Ser) cause consequential abnormal enzymatic activity, and these lead to vitamin-K-dependent protein defects and severe bleeding disorders[37,38]. However, any association between common variants of the GGCX gene with diseases has not been investigated. In 2007, a group of Japanese scientist[34], found another SNP in this gene with associated to BMD in elderly Japanese women, the CCGX with amino acid substitution at position 325 (Arg325Gln) or *rs699664*. The distribution frequency of this SNP found in Japanese population are AA = 9%, AG = 44% , and GG = 47% [39].

Expected benefits:

Usually the studies about efficacy of menatetrenone (MK-4) by using BMD are still controversy. There are some studies have shown the slightly increasing of BMD, unfortunately, mostly of them are not, especially in Caucasian. However, menatetrenone usually maintain the BMD in most of the studies in Japanese population as well as in Thailand. On the other hand, the studies using bone turnover response or ucOC levels usually shows impressive results, but still have some studies report no changes in bone turnover markers, especially studies in Caucasian that mostly use vitamin K1 as the investigational drug. So this study will be benefit in:

1. Some differences of response to ucOC and BTMs may be caused by differences in genotype polymorphism. If we can demonstrate these results, we will understand some of the mechanism underlying bone loss in postmenopausal osteoporosis.
2. Differences in response of the treatment by menatetrenone between Caucasian and Asian may be clarified by differences in genotype distribution of this *GGCX* gene and may open-up our new knowledge about using menatetrenone and other forms of vitamin K in treatment of postmenopausal osteoporosis in Thai population.
3. This is the first time in Thailand and also in Asia to study the differences between *GGCX* gene polymorphism and the responses of ucOC and BTMs. This is also

the first time to find the distribution frequency of *GGCX* gene in Thai postmenopausal population.

Research Conceptual Framework

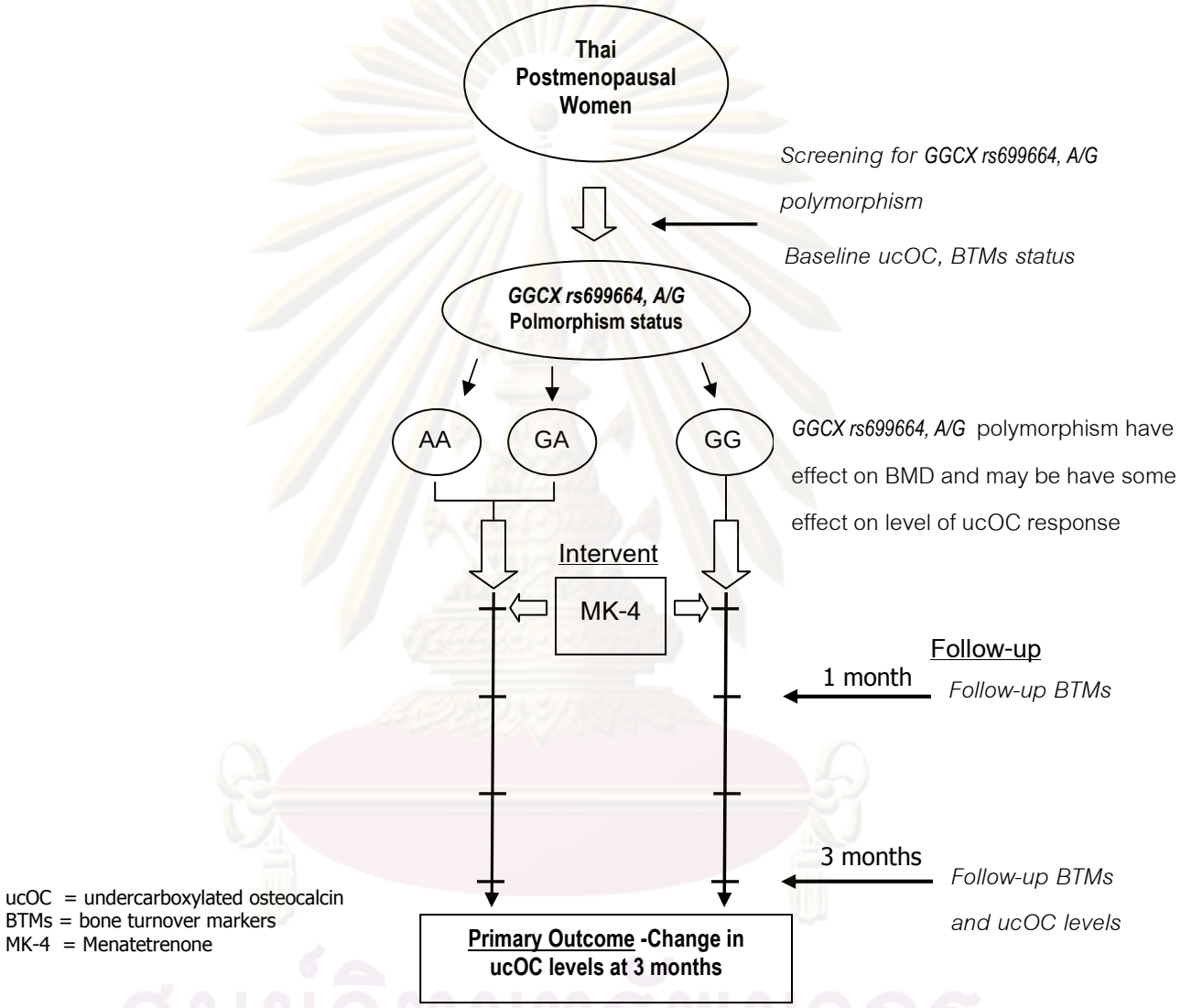


Fig 1.3 Research conceptual framework

Table 1.1 Time schedule

visit	Screening	Day -7 to day 0	Visit 1 (day 0)	Visit 2 (1 month)	Visit 3 (3 months)
- Recruitment - Check for inclusion/exclusion - Informed consent	x				
- Demographic data - Baseline blood testing, ucOC, BTMs, *CCGX* genotyping		x			
- Prescribing MK-4 and prescribing calcium + vitamin D			x		
- Visit and check-up for potential AEs - Prescribing MK-4, calcium + vitamin D - Blood test for BTMs				x	
- Visit and check-up for potential AEs - Prescribing MK-4, calcium + vitamin D - Blood test for ucOC - Blood test for BTMs					x
- Analysis data and prepare manuscript					

CHAPTER 2

Review of Literature

Review of Literature

Many studies about efficacy of menatetrenone (MK-4) by using response in BMD are still controversy. Some studies have shown the increasing of BMD[40-42] in treatment of postmenopausal women, especially in group that combined treatment with vitamin D. Unfortunately, most studies are not. These mostly menatetrenone studies reported maintenance of or suppression of BMD loss at the lumbar spine, hip, or metacarpal regions, or improvements in indices of bone strength in Japanese population[18]. On the other hand, the studies using ucOC levels response usually shows impressive results. Most epidemiologic studies show that the carboxylation status of OC which measured as high serum ucOC level is an independent risk factor for fractures[18] and circulating levels of vitamin K are lower in osteoporotic people with fractures than healthy age-matched controls[43,44]. So, in addition to preservation of BMD, the effect of menatetrenone in most studies which remarkably reduce the serum ucOC levels are potential benefit for reduction of fracture.

To elucidate the mechanism of actions of menatetrenone by using biochemical markers of bone turnover is furthermore controversy.Ushiroyama et al[41], have studied 172 Japanese postmenopausal women who have osteopenia or osteoporosis for 2 years. Subjects were randomized into four groups (each having 43 subjects) which were menatetrenone therapy group, vitamin D3 therapy group, menatetrenone and vitamin D3 combined therapy group, or a control group receiving dietary therapy alone. The bone markers measured in this study revealed stimulation of both bone formation activity by increased in P1CP (type-1 collagen C-terminal propeptide) and intact OC (total osteocalcin) and also increasing in bone resorption activity by increased in urinary pyridinoline. In contrary, Bunyaratavej et al[45], in their study in Thai population on efficacy of menatetrenone in reduction of serum ucOC, were also shown decreasing in serum beta-CTx about 65% in 6 months. This study demonstrates the anti-resorptive property of menatetrenone in corresponding with most in vitro and in vivo studies. Other study in Asian population done by Shiraki et al[46], revealed moderate increase in urinary

NTx in group that received menatetrenone compared with calcium group. In this study, no significant differences of serum BSALP(bone specific alkaline phosphatase) levels which is one of the maker for bone formation between menatetrenone and calcium group throughout the study. There were also no significant differences of urinary DPD (deoxypyridinoline) excretion between the two groups throughout the study. Binkley et al[47], study in 381 healthy postmenopausal North American women randomized into 3 groups, vitamin K1 or phylloquinone (1 mg daily) group , menatetrenone (45 mg daily), or placebo for 12 months. Both phylloquinone and menatetrenone treatment reduced serum ucOC but did not alter serum BSALP or NTX. These variation in efficacy of vitamin K may be due to the type of vitamin K administration (K1 or K2), the difference of absorption and transportation in our body, the tissue specific differences in uptake of vitamin K (K2 is better than K1 in extra-hepatic tissue) and the last reason may be due to differences in efficiency of some enzyme or metabolic pathway which depended on genetic polymorphism.

In study of vitamin K and genetic polymorphism, *GGCX* gene is the most interesting one. It is the gene encoded for enzyme gamma-glutamyl carboxylase. This enzyme is essential for converts glutamate (Glu) residues in the VKD proteins into gamma-carboxyglutamic acid (Gla) residues, and is the vitamin K dependent enzyme which needs vitamin K as a co-factor. The genomic structure of *GGCX gene* was elucidated in 1997[36]. It is reported that the rare mutations of *GGCX gene* with amino acid substitution (Leu395Arg, Trp501Ser) cause consequential abnormal enzymatic activity, and these lead to vitamin-K-dependent protein defects and severe bleeding disorders[37,38].

However, among *GGCX* gene polymorphism, only one SNP reported, found to correlate with BMD[34]. Study by Kinoshita et al [34], shown that the *GGCX* gene is on chromosome 2p12 and the mutation in exon 8 in the position *rs699664* (Arg325Gln) is correlated with BMD. In order to be carboxylated, vitamin K-dependent proteins are assumed to be bound specifically to 343–355 residues of GGCX with high affinity. Among these residues, 343 (Cys) and 345 (Tyr) were suggested to be located near the catalytic center[34]. Moreover, it was also reported that chemical modification of 323-Cys and 343-Cys decreased its carboxylase activity[34]. Considering a study of human GGCX

membrane topology, human GGCX probably may span the endoplasmic reticulum membrane 5 times and the interval of fourth and fifth transmembrane region may be composed of amino acids 313–361[34]. Since amino acids 323-Cys, 325-Arg/Gln, 343-Cys, 345- Tyr and 343–355 are involved in the interval of fourth and fifth transmembrane regions (313–361), the amino acid substitution of 325 residue (Arg/Gln) may affect enzymatic activity directly or indirectly through influencing the function of these residues. People with 325-Gln might have a higher efficiency of carboxylation of these proteins with the given status of vitamin K, considering that carboxylase activity of 325-Gln was higher than that of 325-Arg in vitro. The study by Kinoshita et al[34], did not examine the response of ucOC and the BTMs, so it is the question of this recent study.

CHAPTER 3

Research Methodology

Population

Target population: Ambulatory Thai postmenopausal women

Sample population: Those women who visit to the osteoporosis clinic on Phramongkutklao hospital during study period and fulfill the following criteria.

Eligibility criteria:

Inclusion criteria:

- Thai women living as citizen of Thailand, age 40 or more, and
- Having menopause for at least 2 years, and
- Willingness to participate in the study, and ability to read and provide informed consent.

Exclusion criteria:

- History or evidence of other metabolic bone diseases
- History or evidence of medical or surgical malabsorption syndrome
- History of evidence of other chronic medical diseases that will effects the results and the investigators decide to exclude
- History or evidence of allergy to vitamin K
- Not recently use of warfarin
- Presence of cancer within 5 years.
- Evidence of significant renal (serum creatinine > 2.0 mg/ml) or significant liver impairment (AST or ALT > 3 time UNL)
- Recently consumed an excess alcohol(>4 drink per day), or abused drugs
- Prior use of androgen, estrogen, SERMs, calcitonin, vitamin D-analogs, or corticosteroids within 1 months
- Prior use of bisphosphonate, strontium ranelate or teriparatide within 6 months
- Prior use of investigation drug within 3 months

Sample and Allocation

Sample size determination:

Hypothesis testing for difference of two means

μ_C = mean % change from baseline of ucOC in Thai postmenopausal women after treated with menatetrenone and have AA or AG genotype

μ_A = mean % change from baseline of ucOC in Thai postmenopausal women after treated with menatetrenone and have GG genotype

Hypothesis $H_o: \mu_C = \mu_A$

$H_a: \mu_C \neq \mu_A$

Given that α error = 0.05 (two-tailed), $Z_\alpha = 1.96$

β error = 0.2 (power 80%), $Z_\beta = 0.84$

By study of Prof. Narong Bunyaratavej[45]

Average ucOC level in treatment group(n=43) = 10.00 ± 3.82 ng/ml (mean ± SD)

Average ucOC level in control group(n=40) = 10.68 ± 4.44 ng/ml (mean ± SD)

After 3 months of menatetrenone treatment, the ucOC level fall into 5.31 ± 0.89 ng/ml

Pooled SD $s_p = \sqrt{\frac{(n_1-1)s_1^2 + (n_2-1)s_2^2}{n_1+n_2-2}}$

$= \sqrt{\frac{(43-1)(3.82)^2 + (40-1)(4.44)^2}{43+40-2}}$

$= \sqrt{\frac{612.88 + 768.83}{81}} = 4$

Sample size is calculated from this formula:

$$N = \frac{2\sigma^2 [Z_\alpha + Z_\beta]^2}{(\mu_1-\mu_2)^2} = \frac{2 \times (4)^2 \times (1.96+0.84)^2}{(\mu_1-\mu_2)^2}$$

The difference between group with is about 20% should enough (no study before, but in the study by Prof. Bunyaratavej [45] shown difference of ucOC from baseline about 50% at 3 months treatment, and the SD at 3 months is ± 0.89 or around ±1(10%), so difference between genotype of 20% or 2 ng/ml should be appropriated)

$$\mu_1-\mu_2 = \Delta\mu = 2$$

$$N = (2 \times 16 \times 7.84)/ 4 = 63$$

From distribution frequency from Japanese study in HapMap project[39] are AA = 9%, AG = 44% , and GG = 47% , if we divide in AA or AG group and GG group we can have two group with equal size. So the number of subjects per group after account for 15% drop out is 70, and two groups will be **140**

Allocation technique:

At osteoporosis clinic, Thai postmenopausal women who come to attend will be selected according to the inclusion and exclusion criteria. Women who consent for the study will be included. After that, they will be sent to blood testing for baseline biochemistry, bone turnover markers, ucOC level and *GGCX (rs699664, A/G)* genotyping by masked technicians.

All of them will be receive treatment of menatetrenone 45 mg per day in divided doses of 1x3pc, and calcium carbonate 1.2 g plus vitamin D 400 IU per day in divided doses of 1x2pc.

Outcome Measurements

Primary outcome:

- mean % change from baseline of undercarboxylated osteocalcin (ucOC) at 3 months

Undercarboxylated osteocalcin is the fraction of the osteocalcin in the circulation which is not fully carboxylated. In healthy adults, a very small portion of blood clotting factors is undercarboxylated. In contrast, a substantial portion of circulating OC is undercarboxylated. The marker to measure ucOC in this study is Glu OC-EIA kit. This will be assessed with Enzyme-link Immunoassay (EIA) technique (Takara Bio Inc., Tokyo, Japan, CV 4.6–6.7%)[48,49]. There is diurnal variation in serum osteocalcin, which will be peak in early morning as well as other bone turnover markers. We will be draw the blood in the morning around 8.00 a.m. together with serum beta-CTx and P1NP.

Undercarboxylated Osteocalcin measurement

Principle:

The Glu-OC EIA Kit is a solid phase EIA based on a sandwich method that utilizes two mouse monoclonal anti Glu-OC antibodies to detect Glu-OC or undercarboxylated osteocalcin (ucOC) by two-step procedure (fig 3.1). One of the mouse monoclonal anti-ucOC is immobilized onto the microtiter plate and blocked against non-specific binding. Samples and standards are added each wells and incubated. The second step is to wash the plate and to add the second anti-OC labelled with peroxidase (POD). During this incubation, ucOC is bound to anti-ucOC (solid phase) on one side and tagged on the other by POD-anti OC. The reaction between POD and substrate (H_2O_2 and 3,3' ,5,5' tetramethylbenzidine) results in color development with intensities proportional to the amount of ucOC present in samples and standards. The amount of ucOC can be quantitated by measuring the absorbance using an EIA plate reader. Accurate sample concentrations of ucOC can be determined by comparing their specific absorbances with those obtained for the standards plotted on a standard curve (fig 3.2).

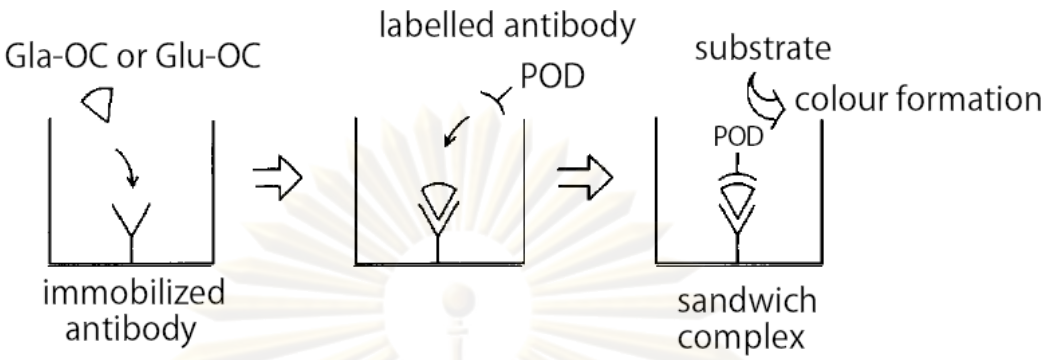


Fig 3.1 Schematic demonstrate enzyme-link Immunoassay (EIA) technique of Gla-OC or Glu-OC(ucOC) kit.

Procedure:

Double determinations of all samples and standards should be performed. All of the Kit's content should be brought to room temperature before use. For thorough mixing, the microtiter plate can be gently agitated on a plate mixer or by mixing the plate sporadically by hand. Enzyme immunoassay was performed using standard procedure according to the manufacturer recommendation. Measure the result absorbance at 450 nm with a plate reader. The absorbance should be read as soon as possible after the completion of the assay. It may be read up to 1 hour after addition of stop solution if wells are protected from light at room temperature.

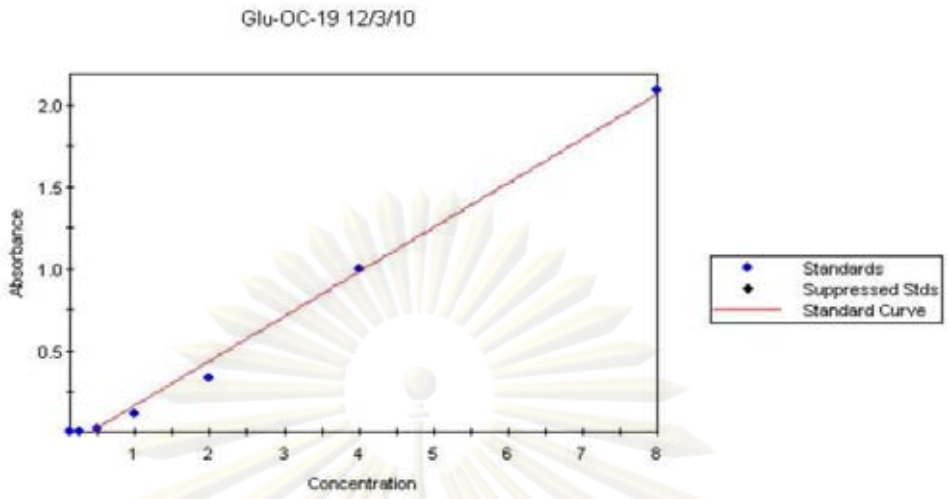


Fig 3.2 Standard curve for measure ucOC

Standard curve (fig 3.2):

Record the absorbance at 450 nm for each standard well. Average the duplicate values and record the averages. Plot the absorbance (vertical axis) versus the ucOC concentration in ng/ml (horizontal axis) for the standards using optimal fitting curve (fig 3.2)

Samples:

Record the absorbance at 450 nm for each sample well. Average the duplicate values and record the averages. Locate the average absorbance value on the vertical axis and follow a horizontal line intersecting the standard curve (fig 3.2). At the point of intersection, read the ucOC concentration (ng/ml) from the horizontal axis.

GGCX genotyping

All subjects' DNA was isolated from frozen buffy coats of whole blood (3 ml, collected into EDTA) by a nonenzymatic extraction method. Sequences of the *GGCX (rs699664, A/G)* gene were amplified, using the polymerase chain reaction (PCR) with primers designed as reported previously[34] (forward primer 5′-TCCTACTGCCCCCGAAGGTTGCAACAA-3′ and reverse primer 5′-TTGTTGCAACCTTCGGGGGCAGTAGGA-3′). PCR cycling conditions and genotyping

will be run by standard procedure according to the endocrine laboratory in Ramathibodi Hospital as following. DNA 20 ng was add into the PCR reaction which consists of TaqMan® Universal Master Mix(1x), TaqMan® MGB probes(1x) in a total volume of 10 μL. The real-time PCR reaction protocol is ,10 min 95° C, and 40 cycles of 15 sec 92° C,1 min 60° C using 7500 Real Time PCR System (Applied Biosystems, Foster City, CA).

Real-Time PCR System

TaqMan® real-time PCR is one of the two types of quantitative PCR methods. Unlike the other type of real-time PCR, the CYBR Green method, which uses a florescent dye that can bind to any double-stranded DNA, TaqMan uses a fluorogenic probe which is a single stranded oligonucleotide of 20-26 nucleotides and is designed to bind only the DNA sequence between the two PCR primers. Therefore only specific PCR product can generate fluorescent signal in TaqMan® PCR. To do TaqMan PCR, besides reagents required for regular PCR, additional things required are a real-time PCR machine, two PCR primers with a preferred product size of 50-150 bp, a probe with a fluorescent reporter or fluorophore such as 6-carboxyfluorescein (FAM) and tetrachlorofluorescin (TET) and quencher such as tetramethylrhodamine (TAMRA) covalently attached to its 5' and 3' ends, respectively.

The probe consists of two types of fluorophores, which are the fluorescent parts of reporter proteins (Green Fluorescent Protein (GFP) has an often-used fluorophore). While the probe is attached or unattached to the template DNA and before the polymerase acts, the quencher (Q) fluorophore (usually a long-wavelength colored dye, such as red) reduces the fluorescence from the reporter (R) fluorophore (usually a short-wavelength colored dye, such as green). It does this by the use of Fluorescence Resonance Energy Transfer (FRET), which is the inhibition of one dye caused by another without emission of a proton. The reporter dye is found on the 5’ end of the probe and the quencher at the 3’ end. Once the TaqMan® probe has bound to its specific piece of the template DNA after denaturation (high temperature) and the reaction cools, the primers anneal to the DNA. Taq polymerase then adds nucleotides and removes the Taqman® probe from the template DNA. This separates the quencher from the reporter,

and allows the reporter to give off its emitted energy. This is then quantified using a computer. The more times the denaturing and annealing takes place, the more opportunities there are for the Taqman® probe to bind and, in turn, the more emitted light is detected.

Secondary outcome:

- mean % change from baseline of bone formation marker P1NP and bone resorption marker beta-CTx at 1 and 3 months
- The pattern of ucOC response in Thai postmenopausal women treated with menatetrenone (MK-4), in descriptive mean and graph
- The pattern of bone turnover markers (BTMs) response in Thai postmenopausal women treated with MK-4, in descriptive mean and graph.
- Distribution frequency of *GGCX (rs699664, A/G)* gene polymorphism (AA, AG and GG genotype) in Thai postmenopausal women, in percent.
- The association between baseline ucOC, bone turnover markers and differences among of *GGCX (rs699664, A/G)* gene polymorphism and will describe in mean and SD.
- mean % change from baseline of bone turnover markers at 1 and 3 months

As we know that bone turnover markers (BTMs) are further divided into the marker of bone resorption and the marker of bone formation, which reflex both two essential part of bone remodeling cycle. The marker of bone resorption use in this study is the serum C-telopeptide cross-linked of collagen type-I (beta-CTx) or beta-Crosslaps assay. This will be assessed with electrochemiluminescence immunoassays (ECLIA) technique (Elecsys, Roche Diagnostics, Mannheim, Germany, ng/ml; intraassay CV 2.4–7.2%)[50]. We also use the marker of bone formation in this study. We choose serum pro-collagen type-I N-terminal propeptide (P1NP), which is the pure bone formation marker and could reflex the bone-stimulating effect of menatetrenone (MK-4). Again, the P1NP assay will be assessed with electrochemiluminescence immunoassays (ECLIA) technique (Elecsys, Roche Diagnostics, Mannheim, Germany, ng/ml; intraassay CV 2.3–2.8%)[51].

Serum for ucOC as well as beta-CTx and P1NP assessment was collected in the morning at around 8.00 am, after an overnight 12 hours fasting and will frozen at –20° C. All specimens will be sent to the central laboratory for test and quality control.

Intervention

Medication:

- Menatetrenone (Glakay®, Eisai Co.,Ltd., Tokyo, Japan) to be taken in one capsule (15 mg) three time daily after meal (1x3pc) for 3 months. Subjects were instructed how to take the drug and checking for compliance by return the drug packet on follow-up date.
- All patients were supplemented daily with 600 mg calcium carbonate combined 200 IU vitamin D at breakfast and lunchtime(1x2pc, totally will have 1.2 g/d of calcium carbonate and 400 IU/d of vitamin D) throughout the 3-month period.
- Concomitant usage of other medications which would affect bone metabolism or other drugs for osteoporosis was prohibited.

Follow-up visit:

- After evaluation for baseline data (visit 1), subjects will received medication as above and we will follow-up in other two visit at 1 month and 3 months
- Visit 2 (1 month ± 1 week after visit 1), the subjects will received the medical evaluation from the investigator and blood testing for serum beta-CTx and P1NP.
- Visit 3 (2 month ± 1 week after visit 2), the subjects will received the medical evaluation from the investigator and blood testing for ucOC and serum beta-CTx and P1NP.

Data Collection

The following data will be recorded:

At baseline:

1) Demographic data, baseline characteristics:
    - Age (year), Weight (kg), Height (cm)

- Years since menopause
- Fracture history
- Other risk factors of osteoporosis

2) Blood testing:
   - Liver and kidney function : serum Cr, AST, ALT, APT, albumin , Ca, P
   - ucOC
   - Bone turnover markers (BTMs) : serum beta-CTx and P1NP
   - *GGCX (rs699664, A/G) gene* polymorphism (AA, AG and GG genotype)

Outcome:

1) Follow-up ucOC and bone turnover markers (BTMs)
   - Bone turnover markers (BTMs) : serum beta-CTx and P1NP at 1 month
   - ucOC and bone turnover markers (BTMs) : serum beta-CTx and P1NP at 3 month
2) Adverse events on follow-up at 1 month and 3 months

Data Analysis

Mean % change from baseline in ucOC level will be calculated and compare before and after treatment within group using paired t-test, and between group between AA, AG genotypes and GG genotype using unpaired t-test.

Mean % change from baseline in bone turnover markers (both serum beta-CTx and P1NP) will be calculated and compare before and after treatment within group, and between group between AA, AG genotypes and GG genotype at 1 month and 3 months using repeated measure ANOVA.

All baseline demographic data and distribution of *GGCX (rs699664, A/G) gene* polymorphism (AA, AG and GG genotype) will be present in percent distribution

We will compare differences between mean ucOC and bone turnover markers between AA, AG genotypes and GG genotype at baseline after adjusted for other baseline characteristics (age, year since menopause,..etc.) by using multiple linear regression

Table 3.1 Summary of measured variables

Data	type of variables	presentation
Demographic data:		
- Age (yr)	continuous numerical	mean, SD
- Weight (kg)	continuous numerical	mean, SD
- Years since menopause	continuous numerical	mean, SD
- Fracture history	categorical , yes/no	N (%)
Baseline variable :		
- Blood testing for liver and kidney function	test for screening and exclude	
- Serum albumin, Ca and P	test for screening and exclude	
- Serum ucOC	continuous numerical	mean, SD
- Serum beta-CTX and P1NP	continuous numerical	mean, SD
- *GGCX (rs699664, A/G)* genotype	ordinal categorical	N (%)
Outcome variables:		
- % change of ucOC from baseline	continuous numerical	mean, SD
- % change of BTMs from baseline (both beta-CTx and P1NP in ng/ml)	continuous numerical	mean, SD

BMD = bone mineral density; BTMs = bone turnover markers
Ca = serum calcium; P = serum phosphorus
ucOC = undercarboxylated osteocalcin assay
beta-CTx = beta-crosslap assay
P1NP = Procollagen type-I N-terminal propeptide assay

CHAPTER 4

Results and Analysis

Subject characteristics

From July 2009 to December 2009, a total of 140 postmenopausal women, who came to Osteoporosis Clinic at Department of Orthopaedics, Phramongkutklao Army Hospital were enrolled to the present study. All of them have been screened for all inclusion and exclusion criteria. They were able to walk independently and none of them engaged in regular physical exercise programs. All of these subjects were never used of bisphosphonate or strontium ranelate or teriparatide before, and have stop all estrogen and SERMs used for more than 1 month as well as for any preparations of calcium and vitamin D supplement.

Table 4.1 Demographic data, baseline characteristics and GGCX distribution frequency in all subjects (140 cases)

		Mean	SD	Min-Max
Age (yr)		59.7	7.2	43-79
Weight (kg)		47.1	5.4	31.5-78.0
Year after menopause (yr)		14.6	6.5	3-30
Serum iPTH (pg/ml)		47.78	4.80	16.28-100.90
Serum ucOC (ng/ml)		2.91	2.07	0.11-9.86
Serum bCTx (ng/ml)		0.47	0.20	0.06-1.02
Serum P1NP (ng/ml)		52.75	21.88	18.32-186.90
GGCX genotype [N (%)]	GG			69 (49.3%)
	GA			66 (47.1%)
	AA			5 (3.6%)

GGCX = gamma-glutamyl carboxylase

The demographic data and baseline characteristics of all these postmenopausal women are shown in table 4.1. There are only 5 cases out of 140 subjects which have previous at least one vertebral compression fracture from lateral plain T-L radiography. None of these subjects has previous hip fracture. The distribution frequency of *GGCX* gene in all these postmenopausal women is shown in table 4.1 and figure 4.1.

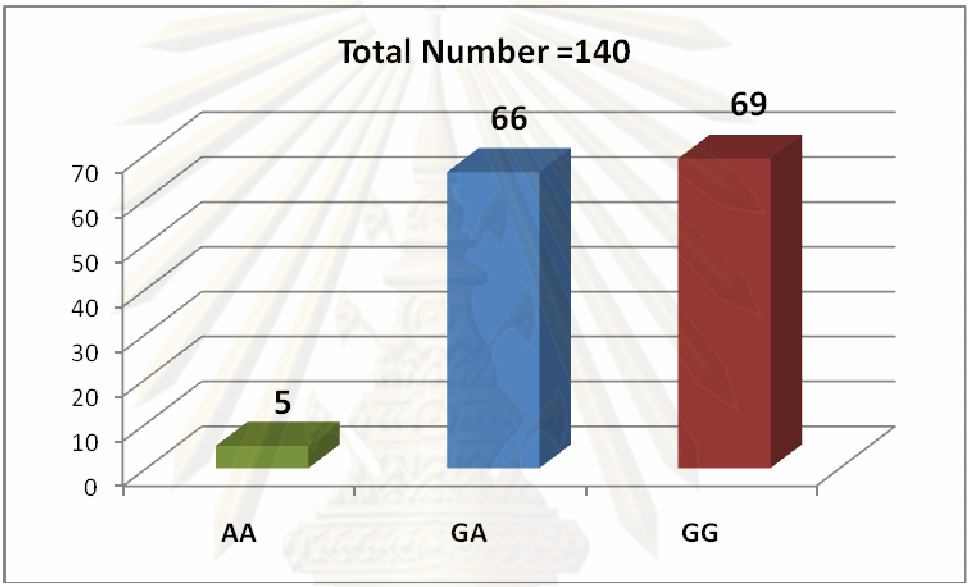


Fig 4.1 Distribution frequency of *GGCX* genotypes

Distribution frequency of *GGCX* genotypes

There are 69 cases (49.3%) of all subjects who have GG genotype (325-Gln), 66 cases (47.1%) of all subjects who have GA genotype (325-Gln/Arg) and only 5 cases (3.6%) of all subjects who have AA genotype (325-Arg) in this present study. In the previous study by Kinoshita et al[34] has grouping the GGCX genotype into two groups of GG and GA+AA, which later shown that there were significant difference in BMI-adjusted Z-score BMD between those who have GG and those who have GA or AA genotypes in subjects that over 75 years of age. We also grouping all these Thai postmenopausal women into two groups as GG-group which included all who have GG

genotype (69 cases) and GA+AA-group which included all who have GA or AA genotype (71 cases). The demographic data and baseline characteristics also divide into two groups as shown in table 4.2. There are no significant difference between these two groups in demographic data and baseline biochemical values.

Table 4.2 Demographic data and baseline characteristic expressed as mean ± SD in all subjects (140 cases) categorized by genotypes.

	GG genotype (N=69)	GA or AA genotype (N=71)	p-value
Age (yr)	60.55 ± 7.51	58.92 ± 6.85	0.18
Weight (kg)	46.98 ± 5.96	47.29 ± 4.86	0.74
Year after menopause (yr)	15.19 ± 6.54	13.99 ± 6.42	0.27
Previous vertebral fractures	2	3	-
Serum iPTH (pg/ml)	46.24 ±15.22	49.27 ± 14.33	0.23
Serum ucOC (ng/ml)	2.92 ± 2.02	2.89 ± 2.14	0.95
Serum bCTx (ng/ml)	0.49 ± 0.21	0.44 ± 0.19	0.17
Serum P1NP (ng/ml)	54.49 ± 25.29	51.06 ± 17.98	0.35

Change in undercarboxylated osteocalcin(ucOC)

There are 14 cases of subjects drop out due to loss of contact in the third visit at 3 months, so the total cases that undertake blood chemistry test for undercarboxylated osteocalcin (ucOC) at the end of study at 3 months are 126 cases. The effect of treatment with menatetrenone and calcium and vitamin D3 on the serum levels of ucOC are significantly reduced in both groups ($p < 0.001$). (figure 4.2). The baseline value of ucOC in each group are 2.92 ± 2.02 ng/mL (mean ± SD) in GG-group, and 2.89 ± 2.15 ng/mL (mean ± SD) in GA+AA-group. The overall reduction in ucOC level in both group are 61.7 percent from baseline and mean % change from baseline in each group are -54.2% and -69.9% in GG-group and GA+AA-group respectively (table 4.3). To compare between the % change for baseline between GG-group and GA+AA-group at 3 months using unpaired t-test, there are no significant difference between GG-group and GA+AA-group ($p = 0.69$).

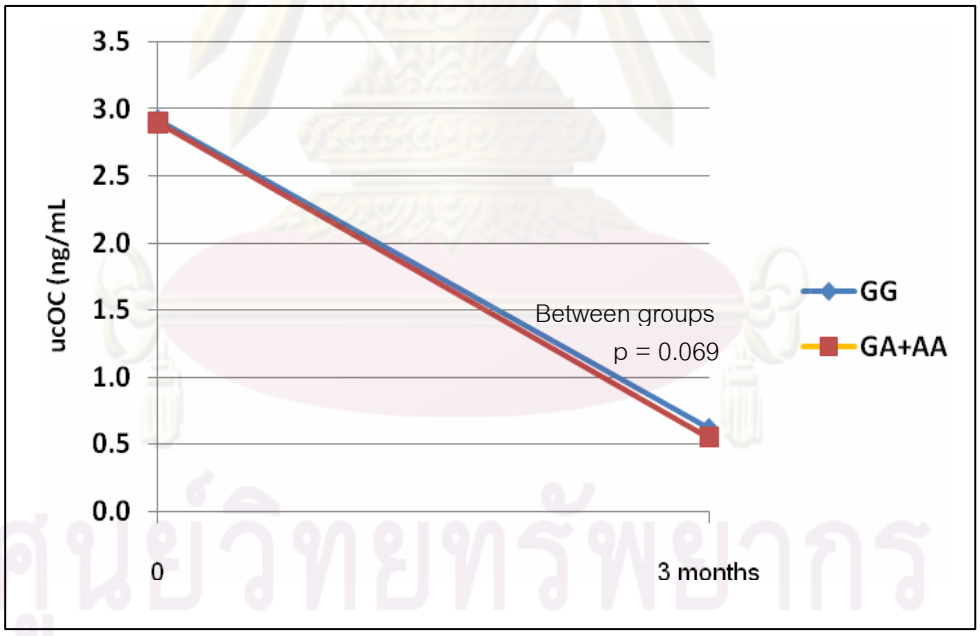


Fig 4.2 Reduction in serum ucOC level from baseline at 3 months

Table 4.3 Mean % change from baseline of serum ucOC at 3 months

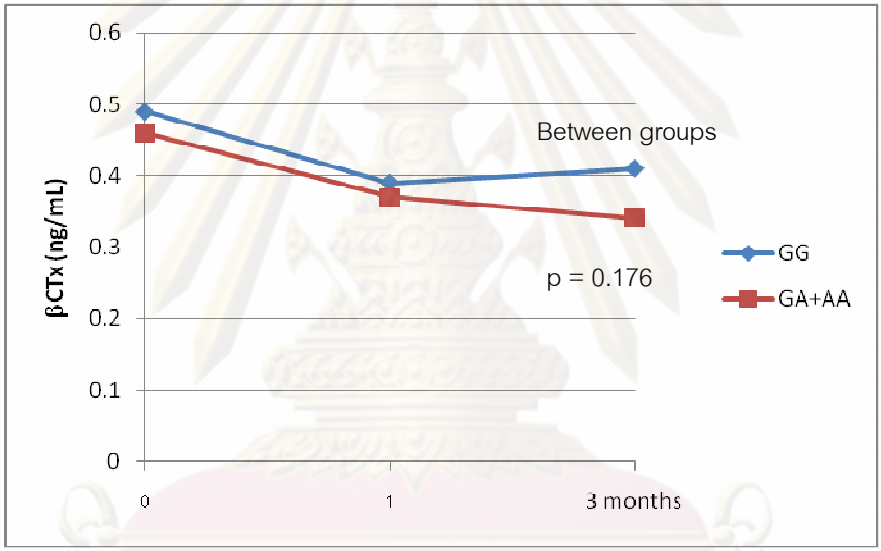
Genotype groups	N	Mean (%)	SD	P-value
Total	126	-61.6967	49.77296	<0.001
GG	66	-54.2244	62.24363	<0.001
GA+AA	60	-69.9163	29.14080	<0.001

Change in bone turnover markers

Along with ucOC study, we performed two bone turnover markers, serum C-telopeptide cross-linked of collagen type-I or beta-crosslaps (beta-CTx) as a representative for bone resorption, and serum pro-collagen type-I N-terminal propeptide (P1NP) as a representative of bone formation. There are 8 cases of subjects that have been dropout from the study at 1 month (N=132) and 14 cases that have been dropout from the study at 3 months(N=126) follow-up. Mean baseline levels of beta-CTx and P1NP are shown in table 4.4. There are a significant reduction in serum beta-CTx and P1NP level from baseline at 1 month and 3 months after treatment with menatetrenone and calcium and vitamin D3 in overall cases and both genotype groups by using one-way repeated ANOVA ($p < 0.001$). The overall mean % reduction in serum beta-CTx and serum P1NP from baseline are -13.8 ± 47.1% and -14.1 ± 20.6%. To compare between two genotype groups, there are no significant different between GG-group and GA+AA-group in both serum beta-CTx and serum P1NP at 3 months shown by figure ($p = 0.176$ and $p = 0.429$ respectively by two-way repeated ANOVA)

Table 4.4 Serum beta-CTx and P1NP at baseline, 1 month and 3 months, all shown in different genotypes and in total cases (mean, N and SD)

Genotype		Baseline βCTx	βCTx at 1 month	βCTx at 3 months	p value	Baseline P1NP	P1NP at 1 month	P1NP at 3 months	p value
GG	Mean	0.4908	0.3950	0.4147	<0.001	54.4964	50.5953	45.2670	<0.001
	N	69	68	66		69	68	66	
	SD	0.2119	0.1720	0.2232		25.2883	24.1211	19.4222	
GA+AA	Mean	0.4449	0.3669	0.3421	<0.001	51.0551	47.2789	43.2862	<0.001
	N	71	64	60		71	64	60	
	SD	0.1878	0.1720	0.1485		17.9785	18.4427	16.6427	
Total	Mean	0.4675	0.3814	0.3798	<0.001	52.7511	48.9873	44.3237	<0.001
	N	140	132	126		140	132	126	
	SD	0.2006	0.1720	0.1937		21.8771	21.5388	18.1073	



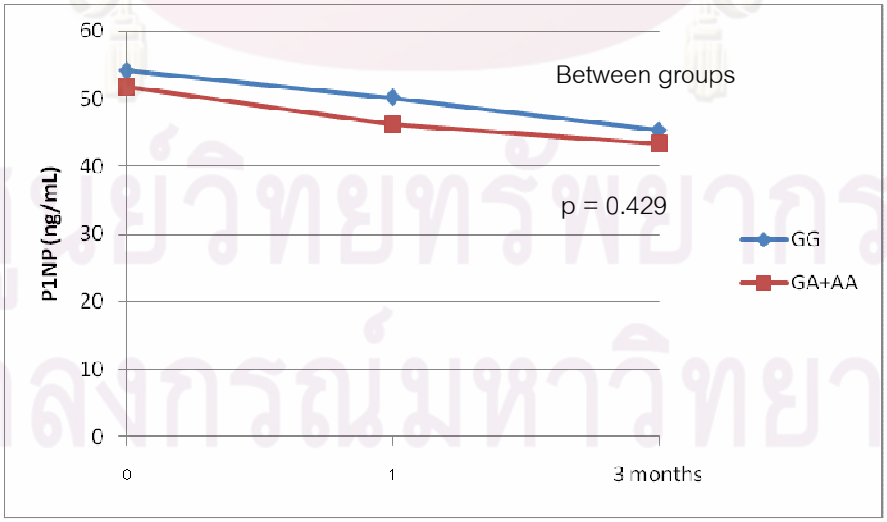


Fig 4.3 Reduction in serum beta-CTx and P1NP level from baseline at 3 months

Analysis of AA genotype group

There are only 5 cases in AA group, which account for 3.6% of all this SNP. One in these 5 cases was loss of contact on visit 3 at 3 months following treatment. All baseline characteristic and % change from baseline of serum ucOC, beta-CTx and P1NP at 3 months of these subjects are shown in table 4.5.

Table 4.5 Baseline characteristics and % change from baseline of serum ucOC, beta-CTx and P1NP at 3 months of all subjects who have AA genotype

	Age	year after menopause	Weight	PTH	Baseline			% change from baseline		
					ucOC	bCTx	P1NP	ucOC	bCTx	P1NP
case 1	55	10	57.0	48.30	3.606	0.424	62.25	-89.21	8.02	-23.94
case 2	55	8	43.0	41.90	0.719	0.392	46.84	-33.10	-47.19	-12.40
case 3	58	13	43.0	43.52	2.477	0.445	70.77	-43.68	-14.83	-11.46
case 4	59	14	45.5	68.99	3.313	0.371	44.84	-70.87	9.97	1.54
case 5	78	30	42.0	47.78	1.664	0.357	48.32	*	*	*
mean	61	15	46.1	50.10	2.356	0.398	54.60	-59.22	-11.01	-11.57
SD	9.67	8.72	6.23	10.91	1.189	0.037	11.35	25.55	26.62	10.42

*= drop out at 3 months due to loss of contact

Adverse events

All study preparations were well tolerated. There were no any serious adverse events in this study. The incidence of non-serious adverse events was 2.8 % (4/140 patients). One case has erythematous skin lash on her extremities on first week of administration which subside by anti-histamine treatment. Other three cases have mild symptom of dyspepsia on first week after administration with spontaneous subsided. All patients with these non-serious adverse events did not dropout from the study. The patients' drop-out in this study are 8 cases on first follow-up visit at 1 month, 7 cases are due to loss of contact, but one case has sustained right ankle fracture 3 weeks after enrollment. On last follow-up visit at 3 months, there were 14 cases of loss follow-up which were 4 cases in GG-group and 10 cases in GA+AA-group. Overall dropout rate at 3 months were 10% (14/140) in this present study.

CHAPTER 5

Discussion and Conclusion

Discussion

Vitamin K, once viewed as having a single physiologic function in blood clotting, is now receiving substantial attention for several biologic effects beyond hemostasis. New research indicates that vitamin K plays a role in bone metabolism and is potentially protective against osteoporosis[52-53]. The effects of vitamin K in bone metabolism involves a similar mechanism of action, that of posttranslationally modifying certain vitamin K–dependent proteins (VKD proteins) for their full biologic activity. Remarkable for bone, it is a protein call osteocalcin (OC) which needs the posttranslational modification to a fully gamma-carboxylated form before completely its function in bone matrix. Osteocalcin was produced by osteoblast under the stimulation of $1,25(OH)_2D_3$[54], which is one of non-calcemic action of vitamin D. Although the precise role of OC in bone metabolism is not fully understood, a number of findings suggest its importance in regulating bone mineralization, maturation, and remodeling[18]. So one of the functions of all analogs of vitamin K (both vitamin K1 and K2) is to enhance bone matrix formation by osteoblast and further improved the quality of bone. In addition to the role of vitamin K in the synthesis of OC, recent research suggests that certain forms of vitamin K may have effects on bone metabolism that are unrelated to OC. In vitro studies demonstrated that menatetrenone or menaquinone-4 (MK-4), a form of vitamin K2, can inhibits bone resorption through inhibition of osteoclast formation and activity[11,12]and induction of apoptosis in osteoclasts[55], whereas vitamin K1 did not. Hence, in preclinical studies, the only vitamin K that might have both functions on improved bone formation and inhibition of bone resorption is menatetrenone (MK-4).

Unfortunately, clinical studies to elucidate the main action of menatetrenone are still complicatedness. Although several clinical trials of menatetrenone have shown that it prevents fractures in patients with osteoporosis [33,56,57], the exact mechanisms of this effect are not fully understood. Since menatetrenone has a minimal influence on BMD [33], explanations for its preventive effect on fracture have focused on improvement of

bone quality[1]. The quality of bone is determined by several factors, including bone architecture and bone turnover, which can be measured in the clinical setting. Recently, Knappen et al.[58] reported that menatetrenone inhibited deterioration of bone architecture in the femoral neck of postmenopausal women. The other bone quality factors that cannot be measured easily in clinical studies are the mineralization condition and quality of bone matrix proteins. As we know that matrix proteins also influence bone properties, several bone matrix proteins such as osteocalcin probably has an important role in determining bone strength. For example, a rapid decrease of bone stiffness after oophorectomy has been observed in osteocalcin knockout mice in one study[59].

Detection of undercarboxylated osteocalcin (ucOC) in the serum is an evidence of skeletal vitamin K insufficiency or deficiency[18], with further result in a decrease of osteocalcin in the bone matrix. A high serum ucOC level or low vitamin K level has been reported to be associated with an increased incidence of femoral neck fracture[26,29] and vertebral fracture[60]. Previous studies have shown that menatetrenone treatment reduces the serum level of ucOC[28,33, 61,62] and a study by Tsugawa et al, revealed that the vitamin K requirement for gammacarboxylation of osteocalcin was greater in older postmenopausal women than in younger women[63]. In this present study, we are not only evaluating the effect of menatetrenone on undercarboxylated osteocalcin, we also evaluate the effect of gene *GGCX* which encode for the enzyme gamma-glutamyl carboxylase (GGCX) in response to menatetrenone. This enzyme is essential for converts glutamate (Glu) residues in undercarboxylated osteocalcin into gamma-carboxyglutamic acid (Gla) residues, and become fully-carboxylated osteocalcin or mature osteocalcin (OC). This enzyme is vitamin K dependent which needs vitamin K as a co-factor in its metabolism. One of the essential SNP of this *GGCX* gene has previous elucidate to have correlated with BMD[34]. The mutation in exon 8 in the position *rs699664* (Arg325Gln) is our interested outcome. Amino acid substitution of 325 residue (Arg/Gln) may affect enzymatic activity directly, and people with 325-Gln (GG) might have a higher efficiency of carboxylation of these VKD proteins with the given status of

vitamin K[34], considering that carboxylase activity of 325-Gln (GG) enzyme was higher than that of 325-Arg (AA) in vitro.

For the primary outcome of this study, we have demonstrate that treatment with the combination of menatetrenone (MK-4) together with calcium and vitamin D3 supplement can dramatically reduce serum level of ucOC in the overall group to about 61.7 percent from baseline and also for both group of GG and GA+AA genotype to about 54.2% and 69.9% from baseline respectively. Surprisingly, there are no significant difference in this reduction between GG-group and GA+AA group. This may be explained by three possibilities. First, there is no significant relationship between this SNP of gene and the GGCX enzymatic activity as contrast to Kinoshita et al, study. The second possibility as previously demonstrated by Kinoshita et al[34], that the kinetic study and reaction rate for carboxylation of the 325-Arg enzyme(AA), was about 1.2-fold slower than that of 325-Gln (GG), it turnout to explain that the 325-Gln(GG) enzyme has higher carboxylase activity than 325-Arg (AA)[34]. They also concluded that for *GGCX* polymorphism, the allele "G" is dominant[34]. In our recent study, the distribution frequency of *GGCX* gene are 49.3% for GG genotype (325-Gln), 47.1% for GA genotype (325-Gln/Arg) and only 3.6% for AA genotype. It is a scarcity of subject who have AA genotype. If there are better carboxylation power for allele "G" and it is also a dominant allele, the slower effect of allele "A" will never been shown up in this clinical study. We try to compare the reduction in serum ucOC from baseline between each group of genotypes(GG, GA and AA) by non-parametric statistics. The result also comes out into no significant difference between GG-group and AA-group.($p = 0.627$, Mann-Whitney U-test). The graph plot by three groups as shown in figure 5.1 demonstrate the tendency that AA genotype group has slower effect on reduction of serum ucOC, but it is difficult to conclude that GG genotype has better effect on reduction of serum ucOC than the other genotype. We need further study that enrolls subjects who have AA genotype farther more than this study to possibly demonstrate the effect. Other effects that may also have influence to this study are the dietary factors[18]. In this study, we could not control for the diets of our ambulatory patients. There may be some differences in habitual food around different region in Bangkok and in Thailand. The dissimilarities in

natural vitamin K1 contents in those foods can cause some effects to our result. The individual gastrointestinal absorption quality on vitamin K may be causes some effects to our study too[18].

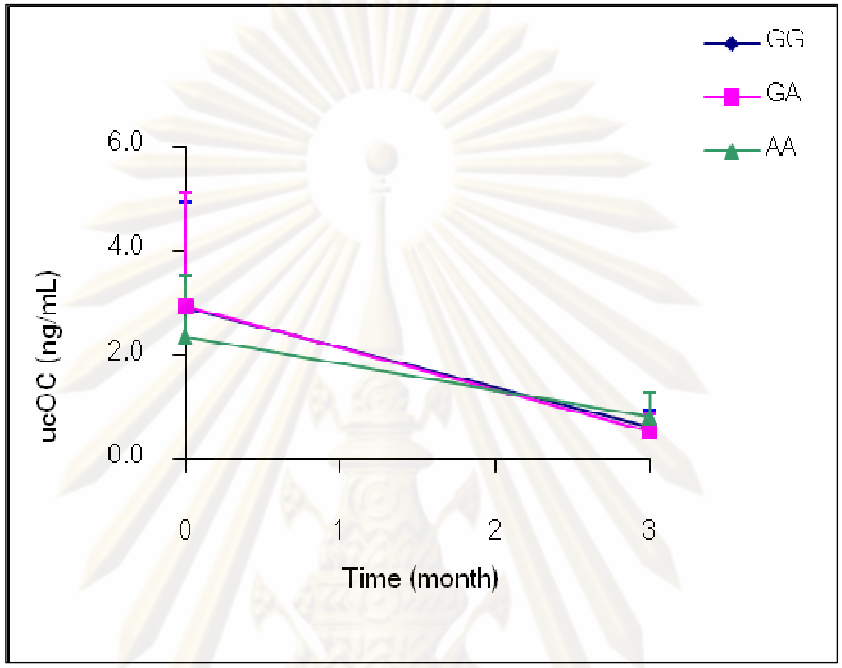


Fig 5.1 Reduction in serum ucOC level from baseline at 3 months in 3 groups of genotypes

We also study the bone turnover response for treatment with menatetrenone (MK-4) concurrently with calcium and vitamin D3 supplement. In this recent study, there are reduction in serum beta-CTx and serum P1NP from baseline in both group of genotype (GG-group and GA+AA-group) significantly. These significant reductions are presented as early as 1 month post-administration. Mean percent change from baseline are not quite large in both markers, there are about 14% in reduction in both serum beta-CTx and serum P1NP at 3 months. These reductions are very small as compared to previous Thai population study[45]. Even there are statistical significant reduction in both markers, these reductions are not so clinically significant at 3 months, compare to other strong anti-resorptive agents such as bisphosphonates. To compare the reductions between two groups of genotypes, there are no significantly different between GG-

group and GA+AA group in both serum beta-CTx and serum P1NP. Although menatetrenone is considered to be the dual action drug, which perform both bone formation improving and inhibition of bone resorption, the decrease in bone resorption marker, serum beta-CTx is the dramatically hallmark of anti-resorptive agent as also demonstrated in previous in vitro studies[11,12,55]. These reductions in serum beta-CTx are not difference in variation of *GGCX* polymorphism. It supports that GGCX enzyme might not have an effect on mechanism of anti-resorption. In the same way, reductions in serum P1NP, which is the marker of bone formation, are not differences between variations in *GGCX* genotypes. The measurement of P1NP is to detect a part of pro-collagen type-1 which has to be cut before secreted out of osteoblasts. These reductions in serum P1NP revealed that one of the bone formation improving mechanisms for menatetrenone might not affect by GGCX enzyme. The important confounding factors to these results are the differences in food intake and the differences of absorption and transportation of vitamin K in our body.

Conclusion

Treatment of menatetrenone in concurrent with calcium and vitamin D promote the gamma-carboxylation of osteocalcin, followed by an decrease of undercarboxylated osteocalcin. There are no significant difference between group of GGCX genotypes in reduction of serum ucOC levels, demonstrate that this SNP may not the key polymorphism for the different in enzymatic effects, or the “G” allele, which is the dominant allele has overcome the effect of this enzyme in mostly of the population. The decreasing in serum βCTx and serum P1NP are demonstrated the anti-resorptive effect of this agent. However, the improving of bone quality by this agent is showing by a decreasing in serum ucOC, which mean that the level of the mature osteocalcin will increase and improved the quality of bone matrix. Further investigations are needed to clarify the mechanism of actions of menatetrenone on bone remodeling as well as the effect of this gene or any other genes, which cause the differences in response to this drug.

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