# รายงานฉบับสมบูรณ์

# Vitamin D receptor gene และ estrogen receptor gene alleles กับบทบาทในการทำให้เกิดโรคกระดูกพรุนในคนไทย

โดย
ผู้ช่วยศาสตราจารย์ นายแพทย์บุญส่ง องค์พิพัฒนกุล เมธีวิจัย สกว.
คณะแพทยศาสตร์ โรงพยาบาลรามาธิบดี มหาวิทยาลัยมหิดล

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# สารบัญ

**โครงการ** Vitamin D receptor **และ** estrogen receptor gene polymorphism **กับบทบาทต่อโรคกระดูกพรุนในคนไทย โดย ผู้ช่วยศาสตราจารย์บุญส่ง องค์พิพัฒนกุล**

**วัตถุประสงค์**

1. **ศึกษาถึง** Bsm1 vitamin D receptor genotype distribution **ในสตรีไทยที่มีและไม่มีโรคกระดูกพรุน**
2. **ศึกษาถึงบทบาททางสรีรวิทยาของ** Bsm1 vitamin D receptor gene polymorphism **โดยดูผลตอบสนองต่อ** 1,25-(OH)2 vitamin D **ในผู้ที่มี** vitamin D receptor gene polymorphism **ต่างกัน**
3. **ศึกษาความสัมพันธ์ระหว่างระดับ** estrogen receptor gene alleles **กับมวลกระดูกในเพศหญิงและชาย**
4. ศึกษาความสำคัญของ estrogen ต่อมวลกระดูกในเพศชาย

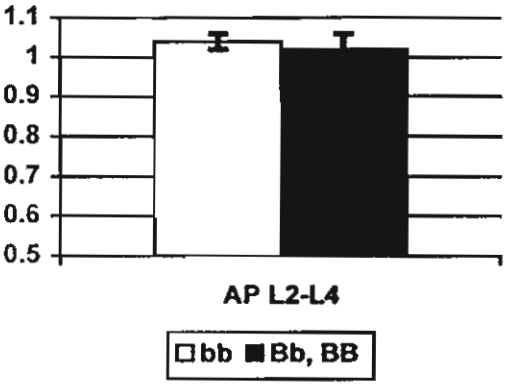
การศึกษาแบ่งออกเป็น 4 โครงการย่อยได้แก่

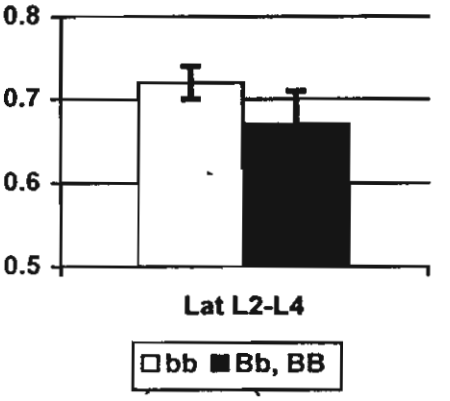
1. Vitamin D receptor gene polymorphism **และ ความหนาแน่นของกระดูกในคนไทย**
2. **ผลตอบสนองต่อ** calcitriol **ในหญิงไทยที่มี** vitamin D receptor gene polymorphism **ต่างกัน**
3. Estrogen receptor gene polymorphism **และ ความหนาแน่นของกระดูกในหญิงไทย**
4. **บทบาทของ** estrogen **และ** estrogen receptor gene polymorphism **ต่อมวลกระดูกในชายไทย**
5. **ผลของการได้รับ** estrogen **ขนาดสูงต่อมวลกระดูกในชาย**

**ผลการศึกษา**

ผลการศึกษาในแต่ละโครงการมีดังนี้

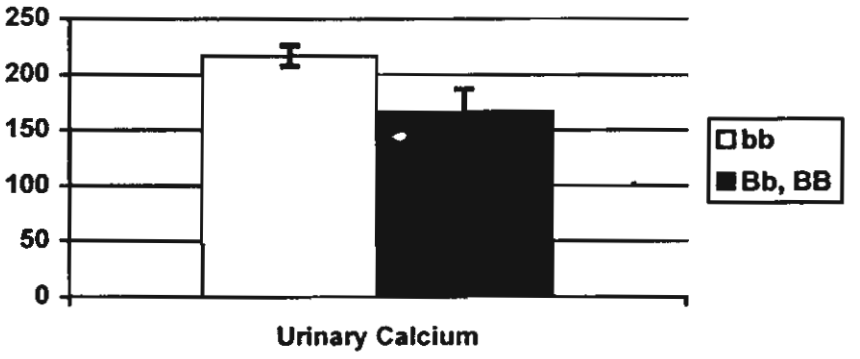
***1. Vitamin D receptor gene polymoprhism และ ความหนาแน่นของมวลกระดูกในคนไทย***




Gene frequency ของ B allele มีค่าน้อยมากในคนไทย และไม่พบมีความสัมพันธ์ระหว่าง vitamin D receptor gene polymorphisms และความหนาแน่นของกระดูกที่บริเวณต่างๆ

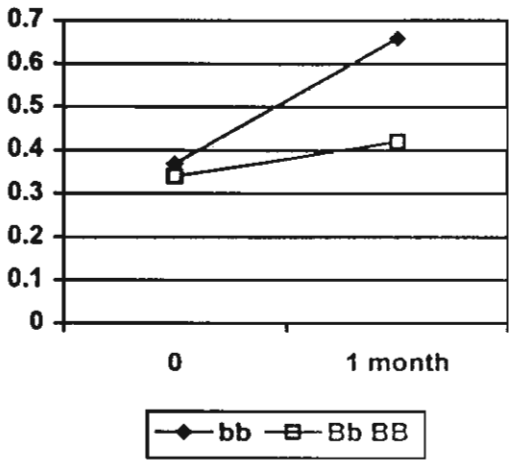
ในด้านดัชนีการทำงานของเซลล์กระดูก ไม่พบว่ามีความแตกต่างกันอย่างมีนัยสำคัญทางสถิติของ serum intact osteocalcin ระหว่างผู้ที่มี vitamin D receptor gene polymorphism ต่างกัน



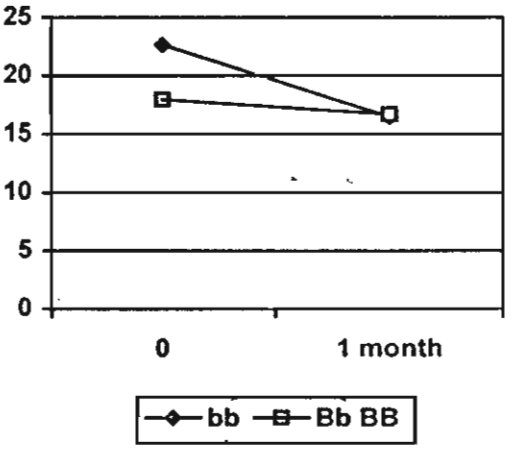

อย่างไรก็ตาม พบความสัมพันธ์อย่างมีนัยสำคัญทางสถิติระหว่างปริมาณแคลเซียมในปัสสาวะ 24 ชั่วโมงและ Bsm1 polymorphism ของ vitamin receptor gene แต่ไม่พบความสัมพันธ์ดังกล่าวใน Apa1 polymorphism ของ vitamin D receptor gene ดังตารางที่ 4 และ 5

ความสัมพันธ์ดังกล่าวในกรณีของ Bsm1 polymorphism ยังคงอยู่หลังจากได้ทำการปรับทางสถิติสำหรับ อายุ ปริมาณแคลเซียมในอาหาร โดยมีค่า $P < 0.05$

***2. ผลตอบสนองต่อ calcitriol ในผู้ที่มี vitamin D receptor gene polymorphism ต่างกัน***




Urinary Calcium Excretion




Parathyroid Hormone

ถึงแม้ vitamin D receptor gene polymorphism จะไม่มีความสัมพันธ์กับความหนาแน่นของกระดูกในคนไทย แต่ผู้ที่มี vitamin D receptor gene polymorphism ต่างกันจะตอบสนองต่อ calcitriol ต่างกัน โดยการให้ calcitriol แก่สตรีวัยหมดประจำเดือนพบว่า ผู้ที่มี genotype ชนิด bb จะมี urinary calcium excretion และ parathyroid hormone ลดลงอย่างมีนัยสำคัญทางสถิติ ส่วนผู้ที่มี genotype ชนิด Bb หรือ BB ไม่พบมีการเปลี่ยนแปลงในดัชนีเหล่านี้

### *3. Estrogen receptor gene polymorphism และความหนาแน่นของกระดูกในหญิงไทย*

| | pp | Pp | PP | Correlation coefficient | P value |
|---|---|---|---|---|---|
| AP L2-L4 BMD ($gm/cm^2$) | 1.12 ± 0.03 | 1.12 ± 0.02 | 1.24 ± 0.06 | 0.25 | 0.07 |
| Lateral L2-L4 BMD ($gm/cm^2$) | 0.80 ± 0.05 | 0.84 ± 0.02 | 0.91 ± 0.06 | 0.25 | 0.08 |
| Femoral neck BMD ($gm/cm^2$) | 0.79 ± 0.03 | 0.84 ± 0.02 | 0.88 ± 0.05 | 0.27 | < 0.05 |
| Femoral trochanter BMD ($gm/cm^2$) | 0.65 ± 0.03 | 0.73 ± 0.02 | 0.75 ± 0.05 | 0.29 | < 0.05 |
| Ward's triangle BMD ($gm/cm^2$) | 0.69 ± 0.04 | 0.78 ± 0.03 | 0.82 ± 0.05 | 0.30 | < 0.05 |
| Mid radius BMD ($gm/cm^2$) | 0.83 ± 0.01 | 0.83 ± 0.01 | 0.82 ± 0.03 | -0.03 | NS |
| Distal radius BMD ($gm/cm^2$) | 0.37 ± 0.01 | 0.38 ± 0.01 | 0.37 ± 0.02 | -0.01 | NS |
| Intact OC (ng/ml) | 5.4 ± 0.5 | 5.0 ± 0.4 | 4.4 ± 0.6 | -0.19 | NS |
| Urinary calcium/creatinine | 0.22 ± 0.03 | 0.26 ± 0.04 | 0.18 ± 0.03 | -0.03 | NS |

Correlation of ER genotype to BMD, intact OC and urinary calcium/creatinine ratio in premenopausal women. The pp genotype is associated with less BMD at various skeletal sites. There was no correlation of ER genotype to either serum intact OC levels or urinary calcium.

พบว่า polymorphism บริเวณ intron 1 ของ estrogen receptor gene มีความสัมพันธ์กับความหนาแน่นของกระดูกโดยเฉพาะอย่างยิ่งกระดูกบริเวณที่รับน้ำหนักเช่น ที่ spine และ femur ในสตรีก่อนหมดประจำเดือน แต่ไม่พบความสัมพันธ์ดังกล่าวในสตรีหลังหลังประจำเดือน

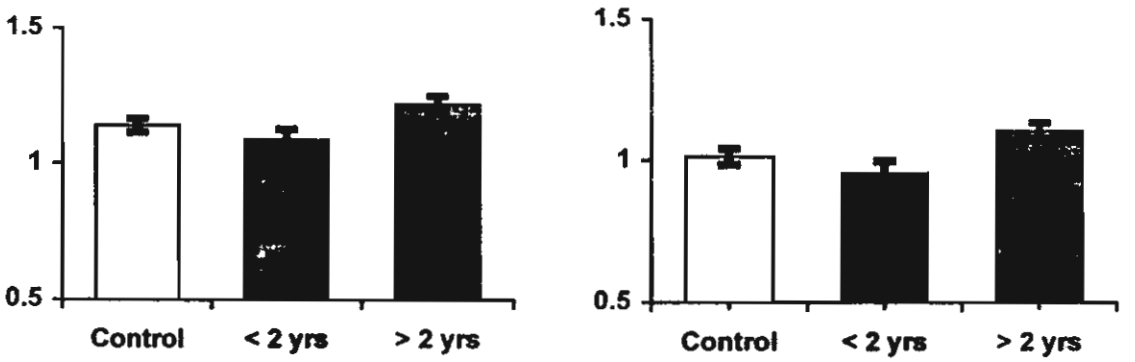
### *4. บทบาทของ estrogen และ estrogen receptor gene polymorphism ต่อมวลกระดูกในชายไทย*

| | Age | BW | Fat | Fat-free | Ca | FT | E2 |
|---|---|---|---|---|---|---|---|
| **AP L2-L4** | **NS** | **NS** | **NS** | **< 0.0001** | **NS** | **NS** | **< 0.05** |
| **Lat L2-L4** | **< 0.01** | **NS** | **NS** | **< 0.0001** | **NS** | **NS** | **NS** |
| **Femoral neck** | **< 0.0001** | **NS** | **NS** | **< 0.05** | **NS** | **NS** | **< 0.01** |
| **Femoral trochanter** | **NS** | **NS** | **NS** | **< 0.05** | **NS** | **NS** | **< 0.05** |
| **Ward** | **< 0.0001** | **NS** | **NS** | **NS** | **NS** | **NS** | **< 0.01** |
| **Mid radius** | **NS** | **NS** | **NS** | **< 0.001** | **NS** | **NS** | **NS** |
| **Distal radius** | **NS** | **NS** | **NS** | **< 0.001** | **NS** | **NS** | **NS** |

ระดับ serum testosterone ลดลงเมื่ออายุมากขึ้น แต่ไม่พบการลดลงดังกล่าวของระดับ estradiol ในเพศชาย เมื่อทำการวิเคราะห์ด้วยวิธี multivariate analysis พบว่า fat-free mass มีความสัมพันธ์กับความหนาแน่นของกระดูกหลายบริเวณ และ ระดับ estrodiol มีความสัมพันธ์กับความหนาแน่นของกระดูกที่บริเวณ AP spine และ femur ในขณะที่ free testosterone ไม่มีความสัมพันธ์กับความหนาแน่นของกระดูกที่บริเวณใดเลย

การกระจายตัวของ estrogen receptor gene ในชายไทยกลุ่มที่ทำการศึกษา พบว่ามี genotypes ชนิด pp 27%, Pp 42% และ PP 14.8% และเมื่อทำการวิเคราะห์ด้วยวิธี multivariate analysis โดยรวม estrogen receptor genotype ด้วย พบว่า estrogen receptor genotype มีความสัมพันธ์กับความหนาแน่นของกระดูกที่บริเวณ AP spine โดยผู้ที่มี p จะมีความหนาแน่นของกระดูกน้อยกว่า ($P < 0.05$)

*5. ผลของการได้รับ estrogen ขนาดสูงต่อมวลกระดูกในชาย*




**AP L2-L4** **Total Femur**

ทำการศึกษาในชาย transsexual ที่ใช้ estrogen ขนาดสูง พบว่า กลุ่มที่ใช้ estrogen เป็นเวลามากกว่า 2 ปี มีความหนาแน่นของกระดูกมากกว่ากลุ่มชายปกติ และกลุ่มที่ใช้ฮอร์โมนน้อยกว่า 2 ปี อย่างมีนัยสำคัญทางสถิติ ถึงแม้ว่าชายเหล่านี้จะมีขนาดของ testicles เล็กเมื่อเทียบกับชายปกติ แสดงว่า estrogen สามารถทำให้มวลกระดูกในชายเพิ่มมากขึ้นได้ แม้ในภาวะที่มี androgen ต่ำ

**ผลงานตีพิมพ์**

การศึกษาในโครงการต่างๆ ได้รับพิจารณาลงตีพิมพ์ กำลังอยู่ในระหว่างพิจารณา และจะส่งตีพิมพ์ดังนี้

1. Publication ในวารสารวิชาการระดับนานาชาติ

1. Ongphiphadhanakul B, Rajatanavin R, Chanprasertyothin S, Chailurkit L, Piaseu N, Teerarungsikul K, Sirisriro R, Komindr S, Puavilai G. Vitamin D receptor gene polymorphism is associated with urinary calcium excretion but not with bone mineral density in postmenopausal women. Accepted for publication in Journal of Endocrinological Investigation.

2. ชื่อเรื่องที่คาดว่าจะตีพิมพ์ได้ วารสารที่คาดว่าจะตีพิมพ์และจะ submit เมื่อใด

1. Ongphiphadhanakul B, Rajatanavin R, Chanprasertyothin S, Chailurkit L, Piaseu N, Sirisriro R, Komindr S. Estrogen receptor gene polymorphism is associated with bone mineral density in premenopausal women but not in postmenopausal women. Submitted to Journal of Endocrinological Investigation.
2. Ongphiphadhanakul B, Rajatanavin R, Chanprasertyothin S, Piaseu N, Cbailurkit L. Serum estradiol and estrogen-receptor gene polymorphism are associated with bone mineral density independent of serum testosterone in normal males. Submitted to Clinical Endocrinology
3. Reutrakul S, Ongphiphadhanakul B, Chanprasertyothin, Kittiyavong S, Piaseu N, Bunnag P, Rajatanavin R. Effect of estrogen exposure on bone mass in male to female transsexuals. Plan to submit to Calcified Tissue International in December 1997.
4. Ongphiphadhanakul B, Chanprasertyothin S, Piaseu N, Rajatanavin R. Responses in bone mass, parathyroid hormone, urinary calcium and bone turnover to calcitriol in subjects with different vitamin D receptor gene polymorphism.. Plan to submit to Clinical Endocrinology in January 1998.

# รายละเอียดของแต่ละการศึกษาในโครงการ

# Vitamin D Receptor Gene Polymorphism is Associated with Urinary Calcium Excretion but not with Bone Mineral Density in Postmenopausal Women

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Running title: Association between vitamin D receptor gene polymorphism and urinary calcium

Keywords: vitamin D receptor, osteoporosis, bone mineral density, genetic polymorphism, osteocalcin.

Supported by the Thailand Research Fund and Anandamahidol Foundation

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## Abstract

Polymorphism of vitamin D receptor (VDR) gene has been found to be associated with serum osteocalcin (OC) levels and bone mineral density (BMD) in Caucasian identical twins and unrelated postmenopausal women. Being ethnically different and living in a geographic area with adequate vitamin D status due to abundant sunshine exposure, it is unclear whether VDR gene polymorphism will affect bone mass in Thai population. In the present study, we investigated the association between VDR gene polymorphism and bone metabolism in Thai postmenopausal women. Subjects consisted of 84 postmenopausal women. Bsm I, Taq I and Apa I polymorphisms of VDR gene were determined by PCR-RFLP. B, T and A represent the absence of the corresponding restriction sites while b, t and a indicate the presence of the restriction sites. Data were expressed as mean ± SEM.

Sixty six (78.6%) of the subjects had bb genotype while 18 (21.4%) had Bb genotype. None of the subjects was found to have BB genotype. Taq I restriction site was in linkage disequilibrium to the Bsm I site. For Apa I polymorphism, 33 (39.3%), 42 (50.0%) and 9 (10.7%) of the subjects had aa, Aa and AA genotypes, respectively. There was no significant difference in serum intact OC levels and BMD at various skeletal sites among subjects with different genotypes. Despite the lack of difference in BMD and intact OC levels, subjects with bb genotype had higher 24-hour urinary calcium excretion than those with Bb genotype (bb, 6.1 ± 0.3 mmol/day; Bb, 4.4 ± 0.6 mmol/day; $P < 0.05$). The effect of Bsm I VDR genotype was still significant ($P < 0.05$) after dietary calcium intake was controlled using analysis of covariance. Despite the difference in urinary calcium levels, there was no significant difference in fractional excretion of calcium among subjects with different Bsm I-related genotypes, suggesting that the effect of the VDR gene polymorphism on urinary calcium excretion is more likely due to the effect on intestinal calcium absorption rather than renal tubular calcium reabsorption.

We conclude that VDR genotype distributions in Thai postmenopausal women are different from those reported in Caucasians. VDR gene polymorphism does not appear to be associated with BMD or bone turnover in Thai postmenopausal women. However, Bsm I VDR polymorphism may have physiologic role in calcium homeostatasis by modulating intestinal calcium absorption.

Bone mass is partly genetically determined (1, 2). It is estimated that as high as 80% of the variance in bone mineral density (BMD) can be attributed to genetic factors (3-6). Recently, polymorphism of vitamin D receptor gene (VDR) was found to be associated with serum osteocalcin (OC) levels and BMD in Caucasian identical twins and unrelated postmenopausal women (7). However, the findings of subsequent studies in different populations were controversial. The reason for the discrepancy is unclear but may be related to different environmental and genetic factors of the studied populations. It is likely that the role of VDR gene in the pathogenesis of osteoporosis and its usefulness as a risk factors for predicting low bone mass may need to be separately evaluated for populations with different genetic backgrounds, lifestyle and environmental factors.

A few studies have evaluated the association of VDR gene polymorphisms and bone mass in Asians (8, 9). Although Asians are more related ethnically, the diet, lifestyles and sunlight exposure in various populations are different. Thailand is geographically situated in an area with abundant sunlight exposure and the vitamin D status of most Thais is sufficient even in the elderly (10). This may influence the effect of VDR receptor gene polymorphism, if there were any. In the present study, we assessed the distribution of VDR gene polymorphism in Thai postmenopausal women and its relation to bone turnover and urinary calcium excretion.

## Subjects and Methods

*Subjects*

Eighty four postmenopausal women aged between 40 and 79 years residing in Bangkok Metropolitan area were recruited by leaflets or direct contact. None of the subjects were smokers or consume significant amount of alcohol. Medical history-taking and complete physical examination were performed on volunteers to assess their health status. All were considered to be healthy and none was taking medications known to influence calcium homeostasis.

All subjects gave informed consent and the study was approved by the ethical clearance committee on human rights related to researches involving human subjects of the Faculty of Medicine, Ramathibodi Hospital, Mahidol University.

*Calcium intake assessment*

Daily calcium intake was determined by a 3-day dietary record in each subject. Subjects were instructed how to keep an accurate 3-day food record. All food items and portions were recorded in the record form for 3 days. Food brand names, methods of food preparation and recipes for any mixed dish eaten during the record period were also included. At the end of the 3-day recording period, they were asked to return the record form for verification of completeness and accuracy by an experienced dietitian at our institute. The subjects were asked to provide additional information about any unclear food item. The computation of calcium intake and nutrient data was done by using the computerized food composition analysis package, "Nutritionist III", modified for Thai foods by the Institute of Nutrition, Mahidol University, Thailand.

*Bone densitometry*

BMD were measured by dual-energy X-ray absorptiometry (Lunar DPX-L, Lunar Corp., U.S.A.). Daily calibration and quality control were done regularly according to the manufacturer's recommendation. The in vitro precision using the spine phantom provided by the manufacturer was 0.6 %. BMD at anteroposterior L2-L4, lateral L2-L4, femoral neck, femoral trochanter, Ward's triangle, distal radius and mid radius were measured in each subject. In vivo coefficients of variation for these sites range from 0.9 % for mid radius to 2.9% for lateral spine.

*Laboratory assays*

Fasting blood samples were obtained from subjects between 8.00 and 10.00 am. Serum samples were frozen at -20 °C until measurement. Serum intact OC was determined by a two-site enzyme immunoassay (Teijin Ltd., Japan).
Twenty-four-hour urine was collected in each subject. Urinary calcium and creatinine were determined by standard methods.

*VDR genotyping*

Genomic DNA was extracted from peripheral leukocytes by phenol/chloroform extraction. Bsm I, Taq I and Apa I polymorphisms of VDR gene were determined by polymerase chain reaction using primers, restriction endonucleases and conditions as previously described (7, 11). The digested PCR products were detected on 1.4% agarose gel with ethidium bromide staining. B, T and A represent the absence of the corresponding restriction sites while b, t and a indicate the presence of the restriction sites.

*Statistical analyses*

Data were expressed as mean ± SEM. Differences among various VDR genotypes were assessed by Student's t test or one-way analysis of variance as appropriate. Analysis of covariance was used to controlled for the effect of age, years since menopause, height, weight and calcium intake on BMD. Adjustment for the effect of dietary calcium on the amount of urinary calcium was also performed by analysis of covariance.

## Results

The genotype distribution of Bsm I, Apa I and Taq I polymorphisms of VDR gene are shown in Table I. It is of noted that BB, and tt genotypes were not present in the studied population. The daily calcium intake in the studied population was 360.5 ± 18.62 mg/day (range 126-1053 mg/day). There was a significant correlation between dietary calcium intake and 24-hour urinary calcium ($r = 0.28$, $P < 0.01$).

There was no significant difference in age, years since menopause, body weight, height and dietary calcium intake among subjects with different VDR genotypes as shown in Table 2. Comparing BMD at various sites, there was no difference at any site among subjects with different genotypes before and after controlling for the effects of age, years since menopause, height, weight and calcium intake (Table 3). Bone turnover as assessed by serum intact OC concentrations was not different in subjects with different genotypes either.

Despite the lack of difference in BMD and bone turnover, subjects with bb genotypes had higher 24- hour urinary calcium than those with Bb genotype (bb, 242.0 ± 12.7 mg; Bb, 174.0 ± 22.1 mg; $P < 0.05$) . After controlling for the amount of dietary calcium intake, the effect of different Bsm I VDR genotypes on urinary calcium was still significant ($P < 0.05$). Fractional excretion of calcium ,as calculated from the ratio of calcium clearance to creatinine clearance, which reflect the role of kidneys in determining serum calcium was not significantly different in those with bb and Bb genotypes (bb, 0.24 ± 0.01; Bb 0.20 ± 0.01). The findings for Taq I polymorphism were similar to those of Bsm I polymorphism. No differences in urinary calcium concentrations or fractional excretion of calcium were found among subjects with different Apa I polymorphism.

## Discussion

Compared to the genotype distributions of VDR gene reported in Caucasian (7, 11), it was apparent that the genotype frequencies in Thais were different. This discrepancy is unlikely to be due to the misclassification of electrophoretic patterns since the previously observed linkage disequilibrium between Bsm I and Taq I polymorphisms was also evident in the present study. However, the results are similar to what have been reported in Japanese (8) and Koreans (9). This suggested that the distribution of VDR gene genotypes may be similar among Asian populations but different from that in Caucasians as suggested in a previous study (12) and the epidemiological impact of the effect of VDR genotypes on bone metabolism in populations with different ethnic backgrounds may be dissimilar.

It has been suggested that one of the genetic determinants of bone mass is VDR gene (7). However, findings from subsequent studies were conflicting in Caucasians as well as in Asians (13). In the present study, although the genotype distributions of VDR gene were similar to those reported in other Asian populations, we could not find any association between VDR polymorphism and BMD at both the axial and the appendicular sites. However, our study was limitted by the absence of BB genotype and the finding was based on the comparison between Bb and bb genotypes of which difference in the effect on BMD may be small, if it were present. The reasons for the discrepancy in findings among different studies are unclear. However, it is likely that other genetic and environmental factors may be contributory. For example, besides VDR gene, mutations of the estrogen-receptor gene and aromatase gene have been reported in patients whose clinical features include osteopenia (14,15). Concerning environmental factors, although the calcium intake in Thais and other Asian populations may be similarly low, Thailand is situated in a geographic area with abundant sunshine exposure and Thai elderly living in Bangkok are not vitamin D deficient. This may modulate the effect of VDR polymorphism on bone mass.

Although there was no difference in bone mass between those with Bb and bb genotypes in the present study, there was significant difference in urinary calcium excretion. Since there was an association between calcium intake and urinary calcium, we also analyzed the data after controlling for the amount of calcium intake. After controlling for dietary calcium, the the effect of Bsm I-related VDR genotypes was still significant suggesting that Bsm I-related VDR genotypes per se may contribute to the difference in urinary calicium excretion which may be attributable to VDR-mediated change in intestinal calcium absorption, bone resorption or calcium reabsorption at the renal tubules. 1,25-dihydroxyvitamin D acts through VDR to increase a

calcium-binding protein (CaBP) and calcium absorption in the intestinal epithelium (16). Moreover, distal renal tubules also contain CaBP (17). However, the precise vitamin D -regulated reabsorption of calcium at the renal tubules is still not well defined although there was evidence localized 1,25-dihydroxyvitamin D receptor to the medullary and cortical thick ascending limbs of Henle's loop in the distal convoluted tubules (18). Despite the change in urinary calcium, there was no difference in fractional excretion of calcium between subjects with bb and Bb genotypes in the present study. This suggested that the observed effect on urinary calcium was less likely to be due to the action of vitamin D endocrine system on renal tubules but was more likely to be caused by the difference in intestinal calcium absorption as has recently been described in a short-term study (19). Together with other reported functional differences among Bsm I vitamin D receptor gene polymorphism such as acute response to 1,25-dihydroxyvitamin D in terms of the suppressibily of PTH and the stimulation of osteoblast (20), Bsm I VDR gene polymorphism may partially affect calcium and bone metabolism. However, the end result in terms of bone mass may be less apparent depending on the effects of other coexisting environmental and genetic factors.

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| | Genotype frequencies n (%) | | |
|---|---|---|---|
| Polymorphism | 1 | 2 | 3 |
| Bsm I | 66 (78.6) | 18 (21.4) | 0 |
| Taq I | 0 | 14 (16.7) | 70 (83.3) |
| Apa I | 33 (39.3) | 42 (50.0) | 9 (10.7) |

Table 1 Genotype frequencies of VDR receptor gene in 84 Thai postmenopausal women. 1 = bb, tt or aa; 2 = Bb, Tt or Aa; 3 = BB, TT or AA.

| | Bsm I | | | Taq I | | | Apa I | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Bb | bb | P | TT | Tt | P | AA | Aa | aa | P |
| Age (year) | 60.0 ± 2.0 | 60.5 ± 1.0 | NS | 57.9 ± 2.0 | 60.9 ± 1.0 | NS | 61.8 ± 2.5 | 59.6 ± 1.3 | 61.0 ± 1.5 | NS |
| Body weight (kg) | 58.3 ± 2.2 | 57.0 ± 0.9 | NS | 57.3 ± 1.0 | 57.3 ± 1.9 | NS | 61.2 ± 3.7 | 56.9 ± 1.1 | 56.7 ± 1.3 | NS |
| Height (cm) | 151.3 ± 1.4 | 154.2 ± 0.4 | NS | 153.7± 0.6 | 153.0 ± 1.4 | NS | 152.0 ± 2.0 | 153.9 ± 0.9 | 153.6 ± 0.9 | NS |
| Years since menopause (year) | 12.7 ± 3.2 | 11.8 ± 1.1 | NS | 12.3 ± 1.1 | 10.6 ± 2.9 | NS | 17.8 ± 6.1 | 11.0 ± 1.2 | 11.7 ± 1.5 | NS |
| Dietary calcium intake (mg/day) | 314.4 ± 33.3 | 373.1 ± 21.7 | NS | 375.8 ± 21.3 | 284.1 ± 27.3 | NS | 266.6 ± 31.9 | 361.9 ± 25.5 | 384.3 ± 32.6 | NS |

Table 2 Clinical characteristics of subjects with different VDR genotypes.

| | Bsm I | | | Taq I | | | Apa I | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Bb | bb | P | TT | Tt | P | AA | Aa | aa | P |
| AP L2-L4 BMD ($g/cm^2$) | 0.98 ± 0.04 | 0.95 ± 0.02 | NS | 0.95 ± 0.02 | 1.01 ± 0.03 | NS | 0.99 ± 0.08 | 0.96 ± 0.02 | 0.95 ± 0.02 | NS |
| Lateral L2-L4 ($g/cm^2$) | 0.65 ± 0.04 | 0.62 ± 0.02 | NS | 0.62 ± 0.02 | 0.67 ± 0.04 | NS | 0.65 ± 0.01 | 0.65 ± 0.02 | 0.60 ± 0.02 | NS |
| Femoral neck ($g/cm^2$) | 0.72 ± 0.03 | 0.74 ± 0.01 | NS | 0.73 ± 0.01 | 0.75 ± 0.03 | NS | 0.66 ± 0.03 | 0.74 ± 0.02 | 0.74 ± 0.02 | NS |
| Femoral trochanter ($g/cm^2$) | 0.62 ± 0.02 | 0.63 ± 0.01 | NS | 0.63 ± 0.01 | 0.64 ± 0.02 | NS | 0.58 ± 0.04 | 0.63 ± 0.01 | 0.63 ± 0.02 | NS |
| Ward's triangle ($g/cm^2$) | 0.58 ± 0.04 | 0.59 ± 0.02 | NS | 0.58 ± 0.02 | 0.61 ± 0.03 | NS | 0.52 ± 0.06 | 0.60 ± 0.02 | 0.58 ± 0.02 | NS |
| Mid radius ($g/cm^2$) | 0.72 ± 0.03 | 0.72 ± 0.01 | NS | 0.71 ± 0.01 | 0.77 ± 0.03 | NS | 0.72 ± 0.03 | 0.72 ± 0.01 | 0.72 ± 0.01 | NS |
| Distal radius ($g/cm^2$) | 0.31 ± 0.01 | 0.31 ± 0.01 | NS | 0.31 ± 0.01 | 0.32 ± 0.01 | NS | 0.30 ± 0.03 | 0.31 ± 0.01 | 0.31 ± 0.01 | NS |
| Intact OC (ng/ml) | 6.4 ± 0.7 | 7.2 ± 0.4 | NS | 7.2 ± 0.4 | 6.5 ± 0.7 | NS | 7.7 ± 1.1 | 6.9 ± 0.5 | 7.0 ± 0.5 | NS |

Table 3 BMD at various skeletal sites in relation to VDR genotypes. No difference in BMD in relation to VDR genotypes was found. No difference in BMD was detected either after controlling for age, years since menopause, height, weight and calcium intake using analysis of covariance

**RESPONSES IN BIOCHEMICAL INDEXES AND BONE MINERAL DENSITY TO CALCITRIOL IS DEPENDENT ON VITAMIN D RECEPTOR GENE POLYMORPHISM IN POSTMENOPAUSAL WOMEN**

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Bone mass is partly genetically determined and vitamin D receptor (VDR) gene has been implicated as one of the contributory factors to bone mass. Polymorphism of the VDR gene has been demonstrated in some, but not all, studies to be related to bone mass. Moreover, VDR gene polymorphism was also shown to be associated intestinal calcium absorption and vitamin D supplement. Since VDR gene frequencies and other ethnic and environmental related factors are different in Thais, we examined the role of VDR polymorphism in modulating responses in calcium and bone metabolism to calcium and calcitriol to assess its modulatory effect in Thais.

Subjects consisted of 34 postmenopausal women. Fourteen had Bb genotypes, 1 with BB genotype and 19 had bb genotype. Sixteen were within 5 years postmenopause and 18 were more than 10 years postmenopause. Sixteen of the subjects were on 0.25 ug calcitriol while 19 were on 0.5 ug calcitriol daily. All subjects also took 750 mg daily supplemental calcium. PTH and intact osteocalcin were measured in fasting serum samples at baseline and 4 weeks after treatment. Urinary deoxypyridinoline (DPD), calcium and creatinine were determined in 24-hour urine samples also at baseline and 4 weeks after treatment. Bone mineral density (BMD) was measured by dual-energy x-ray absorptiometry at baseline and 1 year after treatment.

In subjects with bb genotypes, calcium and calcitriol for 4 weeks caused a decrease in serum PTH (-25.0 ± 7.3%, $P < 0.05$) and an increased in urinary calcium (109.1 ± 28.9%, $P < 0.001$). Serum osteocalcin increased (18.4 ± 5.7%, $P < 0.05$) while DPD remained unchanged (18.5 ± 16.9%). The effects of the bb genotypes on the responses in serum PTH and urinary calcium were dose-independent. Likewise, the effects were also independent of the duration of menopause. In subjects with Bb or BB genotypes, no change in serum PTH (3.2 ± 10.3%), urinary calcium (28 ± 19.7%), osteocalcin (-1.9 ± 4.1%) or DPD (-12.0 ± 10.1%) was demonstrated. After 12 months of treatment, BMD of subjects with bb genotype increased significantly at L2-L4 (11.7 ± 3.6%, $P < 0.01$) while no change was demonstrated in subjects with Bb or BB genotypes (3.6 ± 2%). Such effect of calcitriol on vertebral BMD was not dose dependent nor was it dependent on the duration of menopause. No effect og genotypes on femoral BMD was detected.

We concluded that VDR polymorphism affects both biochemical and skeletal responses to calcium and calcitriol therapy. Assessing VDR genotype may help identify patients more probable to respond favorably to treatment with calcium and calcitriol.

# ESTROGEN RECEPTOR GENE POLYMORPHISM IS ASSOCIATED WITH BONE MINERAL DENSITY IN PREMENOPAUSAL WOMEN BUT NOT IN POSTMENOPAUSAL WOMEN

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Running title: Association of estrogen receptor gene polymorphism and bone mass in premenopausal women

Keywords: estrogen receptor gene, osteoporosis, genetics, bone mass

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**Abstract**

In the present study, we examined the genotypes distribution of *Pvu* II estrogen receptor (ER) gene polymorphism and its association to bone mass in Thai females. Subjects consisted of 134 Thai females of whom 54 were premenopausal and 80 were postmenopausal. *Pvu* II ER gene polymorphism was determined by PCR-RFLP. Capital P represents the absence of the restriction site while small p indicates the presence of the restriction site.

Forty nine (36.6%) of the subjects had pp genotype, while 59 (44.0%) had Pp genotype and 26 (19.4%) had PP genotype. There was no significant difference in age, body weight, height and calcium intake in premenopausal women with different genotypes. The results including years since menopause were similar in postmenopausal women. When including ER gene genotypes, age, body weight, height and dietary calcium intake in a stepwise multiple regression model, it was found that besides body weight ER gene polymorphism was associated with bone mineral density (BMD) at AP spine ($P < 0.05$), lateral spine ($P < 0.05$), femoral neck ($P < 0.05$) and femoral trochanter ($P < 0.05$) with the pp genotype having the least BMD. ER gene polymorphism was the only factor associated with BMD at Ward's triangle ($P < 0.05$) while only body weight was associated with BMD at distal and mid radius. There was no difference in serum intact osteocalcin (OC) concentrations among subjects with different genotypes. ER gene polymorphism was not related to BMD in postmenopausal women at any skeletal site. Similarly, serum intact OC levels were not different among postmenopausal women with different genotypes.

We conclude that *Pvu* II estrogen receptor gene polymorphism is associated with bone mineral density in premenopausal women but not in postmenopausal women. Estrogen receptor gene polymorphism may have modulatory role on calcium and bone metabolism during adolescence and young adulthood.

Although it is generally accepted that bone mass is partly genetically determined (1, 2), it is unclear what the genetic factor is. Candidate gene approach to this problem has initially been explored by Morrison et al. who reported that vitamin D receptor (VDR) gene polymorphism is associated with bone mass in dizygotic twins and unrelated postmenopausal women (3). However, some subsequent studies in different populations yielded different results and the role of VDR gene polymorphisms in bone mass determination remains inconclusive (4). Besides the potential role of VDR gene, it is likely that other candidate genes are also contributory to bone mass determination by affecting either peak bone mass or postmenopausal bone loss. Recently, estrogen receptor (ER) gene mutation has been reported to cause delay in bone maturation and osteoporosis in a male patient (5). This raises the possibility that estrogen endocrine system may play role in bone metabolism during adolescence and ER gene can be considered a potential candidate gene for osteoporosis. In the present study, we investigated whether ER gene plays role in bone mass determination by looking at the association between bone mass in pre- and postmenopausal women with a polymorphism in intron 1 of ER gene.

## Subjects and Methods

*Subjects*

One hundred and thirty four women aged between 20 and 79 years residing in Bangkok Metropolitan area were studied. Fifty four were premenopausal and 80 were postmenopausal. Subjects were recruited by leaflets or direct contact. None of the subjects were smokers or consume significant amount of alcohol. Medical history-taking and complete physical examination were performed on volunteers to assess their health status. All were considered to be healthy and none was taking medications known to influence calcium homeostasis. All subjects gave informed consent and the study was approved by the ethical clearance committee on human rights related to researches involving human subjects of the Faculty of Medicine, Ramathibodi Hospital, Mahidol University.

*Calcium intake assessment*

Daily calcium intake was determined by a 3-day dietary record in each subject. Subjects were instructed how to keep an accurate 3-day food record. All food items and portions were recorded in the record form for 3 days. Food brand names, methods of food preparation and recipes for any mixed dish eaten during the record period were also included. At the end of the 3-day recording period, they were asked to return the record form for verification of completeness and accuracy by an experienced dietitian at our institute. The subjects were asked to provide additional information about any unclear food item. The computation of calcium intake and nutrient data was done by using the computerized food composition analysis package, "Nutritionist III", modified for Thai foods by the Institute of Nutrition, Mahidol University, Thailand.

*Bone densitometry*

Bone mineral densities (BMD) were measured by dual-energy X-ray absorptiometry (Lunar DPX-L, Lunar Corp., U.S.A.). Daily calibration and

quality control were done regularly according to the manufacturer's recommendation. The in vitro precision using the spine phantom provided by the manufacturer was 0.6 %. In vivo coefficients of variation range from 0.9 % for mid radius to 2.9% for lateral spine. BMD at anteroposterior L2-L4, lateral L2-L4, femoral neck, femoral trochanter, Ward's triangle, distal radius and mid radius were measured in each subject.

*Laboratory assays*

Fasting blood samples were obtained from subjects between 8.00 and 10.00 am. Serum samples were frozen at -20 °C until measurement. Serum intact OC was determined by a two-site enzyme immunoassay (Teijin Ltd., Japan).
Twenty-four-hour urine was collected in each subject. Urinary calcium and creatinine were determined by standard methods.

*ER genotyping*

Genomic DNA was extracted from peripheral leukocytes by phenol/chloroform extraction. *Pvu* II polymorphism in intron 1 of the ER gene was determined by polymerase chain reaction using primers, restriction endonucleases and conditions as previously described (6). The digested PCR products were detected on 1.4% agarose gel with ethidium bromide staining. Capital P represents the absence of the restriction site while p indicates the presence of the restriction site.

*Statistical analyses*

Data were expressed as mean ± SEM. Differences among various ER genotypes were assessed by one-way analysis of variance. Pearson's correlation was performed to find the relation between ER genotypes and BMD with genotypes pp, Pp and PP assigned numerical values of 1,2 and 3, respectively. Stepwise multiple linear regression was used to identify variables significantly related to BMD after controlling for other confounding factors.

## Results

Table 1 shows the genotypes distribution in the studied population. No significant difference genotypes distribution was found between pre- and postmenopausal women. There was no difference in age, body weight, height and dietary calcium intake among premenopausal women with different genotypes (Table 2A). For postmenopausal women, there was no difference in these variables and the number of years since menopause either (Table 2B).

In premenopausal women, ER genotypes is associated with BMD at femoral neck, femoral trochanter and Ward's triangle with pp genotype associated with the least BMD. The correlations at anteroposterior and lateral spine approached statistical significance while no correlation between ER genotype and BMD was found at distal and mid radius (Table 3). In terms of bone turnover, although there was a trend for a linear association between serum intact OC levels and ER genotype, this trend did not reach statistical significance. Likewise, there was no correlation between ER genotype and urinary calcium to creatinine ratio. By stepwise multiple linear regression, it was found that body weight was associated with BMD at most skeletal sites. As shown in Table 4, after controlling for the effect of body weight, ER genotype was still significantly associated with BMD particularly at weight-bearing sites

i.e., anteroposterior, lateral spine, femoral neck, femoral trochanter and Ward's triangle. Body weight was associated with BMD at all skeletal sites except at Ward's triangle while age and calcium intake were not associated with BMD at any skeletal site. It is noteworthy that ER genotype was the only factor associated with BMD at Ward's triangle.

There was no correlation between ER genotype and BMD in postmenopausal women (Table 5) . Likewise, there was no difference in intact OC and urinary calcium after corrected for creatinine among subjects with different genotypes. As shown in Table 6, by stepwise multiple regression analyses, the factors strongly associated with BMD were the number of years since menopause and body weight. The number of year since menopause was related to BMD at all measured skeletal sites except mid radius while body weight was associated with BMD at L2-L4, femoral trochanter and radius. In contrast to the finding in premenopausal women, ER genotype was not associated with BMD at any skeletal sites after controlling for other confounding factors (Table 6).

## Discussion

Besides VDR gene, ER gene can be considered a potential candidate gene for osteoporosis. In fact, certain haplotypes as determined by polymorphism to *Xba* I and *Pvu* II restriction endonucleases in intron 1 of the ER gene have been reported to be associate with lower bone mass in postmenopausal women (7). For postmenopausal women in the present study, the number of years since menopause was strongly associated with BMD. However, we did not find an association between bone mass and ER genotypes as determined by *Pvu* II polymorphism in postmenopausal women after controlling for other confounding factors such as the number of years since menopause, age and body weight. This suggests that the effect of ER gene on bone mass in postmenopausal women, if there were any, is likely to be small and less apparent compared to that of estrogen deficiency associated with menopause. However, it is of note that the frequencies of pp genotype in postmenopausal women was higher compared to premenopausal women although this did nof reach statistical significance in terms of genotypes distribution. The genotypes distribution in postmenopausal women also conformed less to Hardy Weinberg equilibrium. The reason for the deviation from Hardy Weinberg equilibrium include new mutation, admixture of different populations and chance sampling. It is more likely that the observed deviation be due to chance sampling and association between ER genotype and BMD in postmenopausal women may be less apparent because of the small sample size.

Genetic factors for bone mass are more likely to affect peak bone mass rather than age-related bone loss. Besides hereditary factors, body weight (8), calcium intake (9, 10) and exercise (11) have also been shown to affect peak bone mass. There appear to be interactions among the various variables in determining peak bone mass. For example, estrogen acts on bone as if to alter the set point to which it adjusts it mass density to mechanical loading (12) and girls entering puberty appears to have 17% higher bone mass compared to prepubertal subjects (13). In the present study, body weight but not calcium intake was related to BMD at various skeletal sites in premenopausal women; and ER genotype was significantly associated with bone mass particularly at weight-bearing sites i.e., spine and femur even after controlling for the effects of body weight. These results in conjunction with the finding of low bone mass in patients with estrogen resistance (5) and aromatase deficiency (14, 15) raise the possibility that ER gene polymorphism may have role in determining peak

bone mass. However, the reason of the association of BMD in premenopausal women with ER gene polymorphism only at weight-bearing site is unclear. It has been suggested that the connecting network of osteocytes in bone may act as mechanostat (16). Moreover, estrogen receptors have been found in osteocytes (17). It is therefore possible that ER polymorphism may act on the osteocytes to modulate their responses to mechanical strain.

It is not readily apparent how intronic polymorphism in the ER gene may affect bone metabolism. There are possibilities that the *Pvu* II polymorphism in the present study may be in linkage to other exonic site in the ER gene or other genes affecting bone metabolism nearby. ER gene polymorphism may affect bone turnover since ER has been found in both osteoblast (18) and osteoclast (19). Although in premenopausal women there was a trend for a relationship between ER gene polymorphism and circulating intact OC levels in the present study, this trend did not reach statistical significance. However, the lack of statistical power because of small sample size remains a possibility. Nevertheless, in a previous study, ER gene polymorphisms also do not affect biochemical markers of bone turnover in Japanese postmenopausal women (7). It is also of note that in that study postmenopausal women with PP genotype had the lowest BMD while premenopausal women with PP genotype in our study had higher BMD. This suggests that ER polymorphism may affect bone metabolism differently according to menopausal status. It is possible that premenopausal women with PP genotype have higher peak bone mass. However, PP genotype also causes more accelerated bone loss after menopause and eventually results in lower bone mass. Whether this is the case needs to be investigated in a prospective manner. Besides direct effects on bone, estrogen also affects intestinal calcium absorption (20) and renal calcium handling (19). However, these two processes are not likely to be major determinants of the observed effects of ER genotypes on BMD since our data showed that there was no association between urinary calcium and ER genotype.

In conclusion, *Pvu* II ER gene polymorphism is associated with BMD in premenopausal women but not in postmenopausal women. This raises the possibility that ER gene polymorphism or other gene nearby may affect calcium and bone metabolism during adolescence and young adulthood. However, the mechanism of this association remains to be determined.

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| Genotype | Genotype frequencies n (%) | | |
|---|---|---|---|
| | pp | Pp | PP |
| Premenopausal | 16 (29.6) | 28 (51.9) | 10 (18.5) |
| Postmenopausal | 33 (41.3) | 31(38.8) | 16(20.0) |
| All | 49 (36.6) | 59 (44.0) | 26 (19.4) |

Table 1 Genotype frequencies of ER gene in 134 Thai women. The genotypes distributions were not significantly different between pre- and postmenopausal women.

| | pp | Pp | PP | P value |
|---|---|---|---|---|
| Age (year) | 37.3 ± 2.2 | 38.6 ± 1.6 | 35.5 ± 2.4 | NS |
| Body weight (kg) | 51.5 ± 1.1 | 52.4 ± 1.6 | 49.5 ± 1.6 | NS |
| Height (cm) | 155.8 ± 1.2 | 155.9 ± 0.8 | 156.9 ± 1.2 | NS |
| Calcium intake (mg/day) | 420.5 ± 50.1 | 381.6 ± 39.7 | 419.9 ± 49.7 | NS |

Table 2A Age, body weight, height and daily calcium intake among premenopausal women with different genotypes.

| | pp | Pp | PP | P value |
|---|---|---|---|---|
| Age (year) | 60.1 ± 1.2 | 61.1 ± 1.6 | 58.2 ± 2.4 | NS |
| Body weight (kg) | 56.1 ± 1.4 | 58.7 ± 1.5 | 56.1 ± 1.9 | NS |
| Height (cm) | 153.6 ± 1.0 | 153.1 ± 0.8 | 153.7 ± 1.4 | NS |
| Calcium intake (mg/day) | 377.7 ± 33.6 | 340.5 ± 30.5 | 368.7 ± 35.9 | NS |
| Years since menopause (year) | 12.1 ± 1.8 | 11.6 ± 1.8 | 12.0 ± 2.5 | NS |

Table 2B Age, body weight, height, daily calcium intake and the number of years since menopause among postmenopausal women with different genotypes.

| | pp | Pp | PP | Correlation coefficient | P |
|---|---|---|---|---|---|
| AP L2-L4 BMD ($gm/cm^2$) | 1.12 ± 0.03 | 1.12 ± 0.02 | 1.24 ± 0.06 | 0.25 | 0.07 |
| Lateral L2-L4 BMD ($gm/cm^2$) | 0.80 ± 0:05 | 0.84 ± 0.02 | 0.91 ± 0.06 | 0.25 | 0.08 |
| Femoral neck BMD ($gm/cm^2$) | 0.79 ± 0.03 | 0.84 ± 0.02 | 0.88 ± 0.05 | 0.27 | < 0.05 |
| Femoral trochanter BMD ($gm/cm^2$) | 0.65 ± 0.03 | 0.73 ± 0.02 | 0.75 ± 0.05 | 0.29 | < 0.05 |
| Ward's triangle BMD ($gm/cm^2$) | 0.69 ± 0.04 | 0.78 ± 0.03 | 0.82 ± 0.05 | 0.30 | < 0.05 |
| Mid radius BMD ($gm/cm^2$) | 0.83 ± 0.01 | 0.83 ± 0.01 | 0.82 ± 0.03 | 0.03 | NS |
| Distal radius BMD ($gm/cm^2$) | 0.37 ± 0.01 | 0.38 ± 0.01 | 0.37 ± 0.02 | -0.01 | NS |
| Intact OC (ng/ml) | 5.4 ± 0.5 | 5.0 ± 0.4 | 4.4 ± 0.6 | -0.19 | NS |
| Urinary calcium/creatinine | 0.22 ± 0.03 | 0.26 ± 0.04 | 0.18 ± 0.03 | -0.03 | NS |

Table 3 Correlation of ER genotype to BMD, intact OC and urinary calcium/creatinine ratio in premenopausal women. The pp genotype is associated with less BMD at various skeletal sites. There was no correlation of ER genotype to either serum intact OC levels or urinary calcium.

| | Age | Body weight | Calcium intake | ER genotype |
|---|---|---|---|---|
| AP L2-L4 BMD | NS | 0.34<br>P < 0.05 | NS | 0.29<br>P < 0.05 |
| Lateral L2-L4 BMD | NS | 0.33<br>P < 0.05 | NS | 0.26<br>P < 0.05 |
| Femoral neck BMD | NS | 0.35<br>P < 0.01 | NS | 0.32<br>P < 0.05 |
| Femoral trochanter BMD | NS | 0.31<br>P < 0.05 | NS | 0.32<br>P < 0.05 |
| Ward's triangle BMD | NS | NS | NS | 0.34<br>P < 0.05 |
| Mid radius BMD | NS | 0.39<br>P < 0.01 | NS | NS |
| Distal radius BMD | NS | 0.38<br>P < 0.01 | NS | NS |

Table 4 Standardized correlation coefficients for the associations of age, body weight, calcium intake and ER genotype to BMD at various skeletal sites in premenopausal women from stepwise multiple linear regression models. ER genotype was significantly associated with BMD at AP spine, lateral spine, femoral neck, femoral trochanter and Ward's triangle after controlling for age, body weight and calcium intake .

| | pp | Pp | PP | Correlation coefficient | P |
|---|---|---|---|---|---|
| AP L2-L4 BMD ($gm/cm^2$) | 0.95 ± 0.03 | 0.98 ± 0.03 | 0.94 ± 0.04 | 0.02 | NS |
| Lateral L2-L4 BMD ($gm/cm^2$) | 0.59 ± 0.02 | 0.67 ± 0.04 | 0.64 ± 0.04 | 0.13 | NS |
| Femoral neck BMD ($gm/cm^2$) | 0.74 ± 0.02 | 0.74 ± 0.02 | 0.72 ± 0.02 | -0.05 | NS |
| Femoral trochanter BMD ($gm/cm^2$) | 0.63 ± 0.02 | 0.63 ± 0.02 | 0.60 ± 0.02 | -0.11 | NS |
| Ward's triangle BMD ($gm/cm^2$) | 0.59 ± 0.03 | 0.60 ± 0.03 | 0.56 ± 0.03 | -0.06 | NS |
| Mid radius BMD ($gm/cm^2$) | 0.71 ± 0.02 | 0.72 ± 0.02 | 0.75 ± 0.02 | 0.15 | NS |
| Distal radius BMD ($gm/cm^2$) | 0.30 ± 0.01 | 0.31 ± 0.01 | 0.32 ± 0.01 | 0.13 | NS |
| Intact OC (ng/ml) | 6.6 ± 0.6 | 7.8 ± 0.5 | 6.8 ± 0.6 | 0.06 | NS |
| Urinary calcium/creatinine | 0.30 ± 0.03 | 0.29 ± 0.02 | 0.24 ± 0.03 | -0.16 | NS |

Table 5 Correlation of ER genotype to BMD, intact OC and urinary calcium/creatinine ratio in postmenopausal women. There was no significant correlation of ER genotype to BMD, serum intact OC levels or urinary calcium to creatinine ratio.

| | Age | Body weight | Calcium intake | ER genotype | Years since menopause |
|---|---|---|---|---|---|
| AP L2-L4 BMD | NS | 0.34<br>P < 0.01 | NS | NS | -0.32<br>P < 0.001 |
| Lateral L2-L4 BMD | NS | 0.36<br>P < 0.001 | NS | NS | -0.41<br>P < 0.001 |
| Femoral neck BMD | NS | NS | NS | NS | -0.57<br>P < 0.001 |
| Femoral trochanter BMD | NS | 0.34<br>P < 0.001 | NS | NS | -0.36<br>P < 0.001 |
| Ward's triangle BMD | NS | NS | NS | NS | -0.55<br>P < 0.001 |
| Mid radius BMD | -0.64<br>P < 0.001 | 0.26<br>P < 0.01 | NS | NS | NS |
| Distal radius BMD | -0.30<br>P < 0.05 | 0.26<br>P < 0.01 | NS | NS | -0.31<br>P < 0.001 |

Table 6 Standardized correlation coefficients for the associations of age, body weight, calcium intake, ER genotype and the numbers of years since menopause to BMD at various skeletal sites in postmenopausal women from stepwise multiple linear regression models. ER genotype was not significantly associated with BMD at any skeletal site.

# SERUM ESTRADIOL AND ESTROGEN-RECEPTOR GENE POLYMORPHISM ARE ASSOCIATED WITH BONE MINERAL DENSITY INDEPENDENTLY OF SERUM TESTOSTERONE IN NORMAL MALES

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## Abstract

OBJECTIVES The physiologic effects of estrogens on bone in men were largely unanticipated until recently; when estrogen deficiency in males with aromatase deficiency and estrogen resistance was found to cause osteoporosis and delayed fusion of epiphyses despite sufficient serum testosterone. This raises the possibility that in normal men estrogens rather than androgens are of physiologic importance in bone maturation. In the present study, we examined the association of serum estradiol (E2) compared to that of free testosterone (FT) with bone mineral density (BMD) in normal men. The effect of estrogen receptor (ER) gene polymorphism on BMD in men was also addressed.

SUBJECTS Subjects consisted of 81 Thai men aged 20-79 years. All were healthy and did not take medication which may affect calcium and bone metabolism. BMD were assessed by DEXA. Dietary calcium was assessed by a 3-day dietary record. Serum E2 and FT concentrations were measured by radioimmunoassay. Polymorphism at intron 1 of the ER gene was determined by PCR-RFLP. Small p represents the presence of the restriction site while capital P indicates the absence of the restriction site.

RESULTS Serum FT decreased with increasing age ($r = -0.58$, $P < 0.0001$) while E2 did not. However, there was a positive association between E2 and FT ($r = 0.28$, $P < 0.05$). Serum FT was related to BMD at femoral neck ($r = 0.26$, $P < 0.05$) and Ward's triangle ($r = 0.30$, $P < 0.01$) while E2 was related to BMD at AP spine ($r = 0.29$, $P < 0.05$), femoral neck ($r = 0.23$, $P < 0.05$) and femoral trochanter ($r = 0.27$, $P < 0.05$). Besides FT and E2, age, body weight, fat mass and fat-free mass were also correlated to BMD at various skeletal sites. Using stepwise multiple linear regression to control for the confounding effects among these factors, fat-free mass was found to be strongly associated with BMD at most skeletal sites. Serum E2 was related to BMD independently of other factors including FT at AP spine ($r = 0.22$, $P < 0.05$), femoral neck ($r = 0.26$, $P < 0.01$), femoral trochanter ($r = 0.22$, $P < 0.05$) and Ward's triangle ($r = 0.26$, $P < 0.01$) while serum FT was not associated with BMD at any site after controlling for E2 and other related factors. Concerning ER gene polymorphism, 27 (33.3%) of the subjects had pp genotype, while 42 (51.9%) and 12 (14.8%) Pp and PP genotypes, respectively. After controlling for age, BW, fat mass, fat-free mass, calcium intake, FT and E2, the presence of p allele was associated with less BMD at AP L2-L4 ($P < 0.05$).

CONCLUSIONS Serum estradiol is more related to bone mass than free testosterone in normal men. Estrogen-receptor gene polymorphism is also associated with bone mass in men independently of estradiol levels. Serum estradiol together with estrogen-receptor genotype may partly determine bone mass in males.

Estrogen deficiency associated with menopause is the main etiologic factor for osteoporosis in women. In men, however, the causes of osteoporosis are more heterogeneous (Orewoll et al., 1995). Although hypogonadism accounts for some cases of male osteoporosis, the etiology in the majority of subjects is less apparent. Both estrogens and androgens affect bone cells. For example, estrogen receptors have been identified in both osteoblasts (Eriksen et al., 1988) and osteoclasts (Oursler et al., 1991) and estrogen deficiency increases bone resorption which is probably mediated through the increase in cytokines production from osteoblasts (Horowitz et al., 1993). In men, androgen deficiency also affects bone mass such that hypogonadal men have reduced bone mass (Goldray et al., 1993). However, whether this is the direct effect of androgens or the indirect action of estrogens produced from the aromatization of androgens is unknown. The effects of estrogens on bone in men is largely unanticipated until recently, when osteoporosis was reports in male patients with estrogen resistance (Smith et al., 1994) and aromatase deficiency (Morishima et al., 1995) despite adequate circulating testosterone. In normal men, however, the relative importance of androgens and estrogens in age-related bone loss is unknown.

Osteoporosis is genetically determined. Nevertheless, what constitutes these genetic factors is largely unknown. It has been suggested that vitamin D receptor gene may be one of the genes for osteoporosis (Morrison et al., 1994). However, most of subsequent studies were not able to confirm the previous findings (Peacock, 1995). Recently, estrogen receptor (ER) gene polymorphism has been implicated as another genetic factors for osteoporosis in women (Kobayashi et al., 1996). How this common ER gene polymorphism may affects bone mass in men is unknown. It was the purposes of the present study to 1) examine the relative contributions of circulating androgen and estrogen on male age-related bone loss and 2) assess the association between the ER gene polymorphism and bone mass in men.

## Subjects and Methods

*Subjects*

Eighty one men aged between 20 and 79 years residing in Bangkok Metropolitan area were studied. Subjects were recruited by leaflets or direct contact. None of the subjects were heavy smokers or consume significant amount of alcohol. Medical history-taking and complete physical examination were performed on volunteers to assess their health status. All were considered to be healthy and none was taking medications known to influence calcium homeostasis. All subjects gave informed consent and the study was approved by the ethical clearance committee on human rights related to researches involving human subjects of the Faculty of Medicine, Ramathibodi Hospital, Mahidol University.

*Calcium intake assessment*

Daily calcium intake was determined by a 3-day dietary record in each subject. Subjects were instructed how to keep an accurate 3-day food record. All food items and portions were recorded in the record form for 3 days. Food brand names, methods of food preparation and recipes for any mixed dish eaten during the record period were also included. At the end of the 3-day recording period, they were asked to return the record form for verification of completeness and accuracy by an experienced dietitian at our institute. The subjects were asked to provide additional information about any unclear food

item. The computation of calcium intake and nutrient data was done by using the computerized food composition analysis package, "Nutritionist III", modified for Thai foods by the Institute of Nutrition, Mahidol University, Thailand.

*Bone densitometry*

Bone mineral densities (BMD) were measured by dual-energy X-ray absorptiometry (Lunar DPX-L, Lunar Corp., U.S.A.). Daily calibration and quality control were done regularly according to the manufacturer's recommendation. The in vitro precision using the spine phantom provided by the manufacturer was 0.6 %. In vivo coefficients of variation range from 0.9 % for mid radius to 2.9% for lateral spine. BMD at anteroposterior L2-L4, lateral L2-L4, femoral neck, femoral trochanter, Ward's triangle, distal radius and mid radius were measured in each subject.

*Laboratory assays*

Fasting blood samples were obtained from subjects between 8.00 and 10.00 am. Serum samples were frozen at -20 °C until measurement. Serum free testosterone (FT) and estradiol (E2) were determined by radioimmunoassay (Diagnostic Product Corp, USA).

*ER genotyping*

Genomic DNA was extracted from peripheral leukocytes by phenol/chloroform extraction. *Pvu* II polymorphism in intron 1 of the ER gene was determined by polymerase chain reaction using primers, restriction endonucleases and conditions as previously described (Kobayashi et al., 1996). The digested PCR products were detected on 1.4% agarose gel with ethidium bromide staining. Capital P represents the absence of the restriction site while p indicates the presence of the restriction site.

*Statistical analyses*

Data were expressed as mean ± SEM. Differences among various ER genotypes were assessed by one-way analysis of variance. Spearmen's correlation was performed to find the relation between ER genotypes and BMD. Stepwise multiple linear regression was used to identify variables significantly related to BMD after controlling for other confounding factors.

## Results

Serum FT decreased with increasing age ($r = -0.58$, $P < 0.0001$) such that at 60 years of age mean serum FT levels decreased to 34% of that at 20 years old (Fig 1A) In contrast to the finding with FT, serum E2 did not decreased with age (Fig 1B) However, there was a positive association between serum E2 and FT ($r = 0.28$, $P < 0.05$) (Fig. 2).

In terms of BMD, serum FT was related to BMD at femoral neck ($r = 0.26$, $P < 0.05$) and Ward's triangle ($r = 0.30$, $P < 0.01$). E2 was related to BMD at anteroposterior L2-4 ($r = 0.29$, $P < 0.05$), femoral neck ( $r = 0.23$, $P < 0.05$) and femoral trochanter ($r = 0.27$, $P < 0.05$). Besides FT and E2, age, body weight, fat mass, fat-free mass were also correlated to BMD at various skeletal sites while calcium intake did not correlated to BMD at any site (Table 1). By stepwise multiple linear regression, fat-free mass was found to be strongly associated with BMD at most skeletal sites. Serum E2 was related to BMD independently of other factors including FT at anteroposterior L2-4 femoral

neck, femoral trochanter and Ward's triangle while serum FT was not associated with BMD at any site after controlling for E2 and other related factors (Table 2).

Concerning ER gene polymorphism, 27 (33.3%) of the subjects had pp genotype, while 42 (51.9%) and 12 (14.8%) Pp and PP genotypes, respectively. Those with p allele had significantly lower spinal BMD but not at other skeletal sites (Fig. 3). After controlling for age, BW, fat mass, fat-free mass, calcium intake, FT and E2, the presence of p allele was still associated with less BMD at AP L2-L4 ($P < 0.05$).

## Discussion

Androgens affect bone metabolism by stimulating human bone cell proliferation and differentiation (Kasperk et al., 1989) which appears to be mediated by transforming growth factor-β, fibroblast growth factor and insulin-like growth factor II (Kasperk et al., 1990). Androgens may act through specific receptors on bone since androgen receptors have been identified on osteoblasts (Colvard et al., 1989). Clinically, hypogonadal men have low bone mass which can be alleviated by androgen replacement (Morley et al., 1993) and a number of studies have found positive relation between circulating testosterone and bone mass (Kelly et al., 1990; Murphy et al., 1993). In the present study, BMD at various sites in normal men was found to be associated with both circulating E2 and FT when univariate analyses were used. However, after the effect of E2 was taken into account, FT was no longer significantly related to BMD. This suggests that most of the observed effects of testosterone on bone are not direct but may be mediated through estrogens derived from androgens. The present finding was in keeping with the demonstration of low bone mass in male subjects with estrogen resistance (Smith et al., 1994) and aromatase deficiency (Morishima et al., 1995) despite high circulating levels of testosterone. In experimental animals, there were also evidences suggesting that estrogens are relatively more important than androgens in the determination of bone mass in males. For examples, orchidectomized rats treated with testosterone together with an aromatase inhibitor did not attain as much bone mass as those treated with estrogen alone (Vanderschueren et al., 1996). Moreover, mice with targeted disruption of ER gene also have low bone mass similar to the findings in the patient with estrogen resistance (Korach et al., 1996). The evidences combined suggest that bone mass in men is more accountable for by estrogens rather androgens. The role of relative estrogen deficiency in the pathogenesis of male osteoporosis remains to be elucidated.

A number of factors may affect bone mass in men besides sex steroids. However, this information is relatively scarce compared to what was known in women. It has been demonstrated that body weight is a strong contributory factors to bone mass in men (Edelstein et al., 1993; Bendavid et al., 1996; Mazess et al., 1996). In terms of body composition, although fat mass is associated with BMD in women, it is not associated with BMD in men (Reid et al, 1992) which was also demonstrated in the present study. Fat free mass appeared to have a stronger association with BMD than fat mass, a finding which was similarly observed in a previous study (Baumgartner et al., 1996). Association between calcium intake and bone mass (Kelly et al., 1990) in men has been described, although we did not find such association in the present study. The reasons for the discrepancy may be related to the amount of daily calcium intake. Since calcium intake in our studied population is relatively low in most subjects, the relative effect of calcium on bone mass may therefore be more difficult to demonstrate. What underlies the observed association of E2

with BMD only at weight-bearing site is unclear. Mechanical load is an crucial factor in the attainment of bone mass and the network of osteocytes may functions as the mechanoceptor in bone. Since estrogen receptor has been found in osteocytes (Braidman et al., 1995), it is possible that estrogens may act on bone to modulated the response of osteocyte to mechanical strain.

BMD in both females and males are partly genetically determined as examined in family (Krall et al., 1993) or twins studies (Smith et al, 1973). Moreover, bone turnover as assessed by serum osteocalcin levels was also genetically related in female twins (Kelly et al., 1991). Although the nature of the inheritance is likely to be polygenic, a few candidate genes have been implicated. For examples, VDR gene polymorphism has been suggested to be related to bone mass (Morrison et al., 1994), although a number of subsequent studies were not able to confirm the previous finding (Peacock, 1995). Recently, ER gene polymorphism was also shown to be associated with bone mass in postmenopausal women (Kobayashi 1996). However, it is unclear whether the finding in males is similar. In the present study, we demonstrated that ER gene polymorphism was related to BMD only at anteroposterior spines after controlling for other covariates. The reason for the association only at anteroposterior spine is unclear but may be related to the site-specific difference in skeletal response (Riggs et al . 1981).

We concluded that serum E2 is more related to bone mass than free testosterone in normal men. ER gene polymorphism is also associated with bone mass in men independently of E2 levels. Serum estradiol together with estrogen-receptor genotype may partly determine bone mass in males.

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## Figures

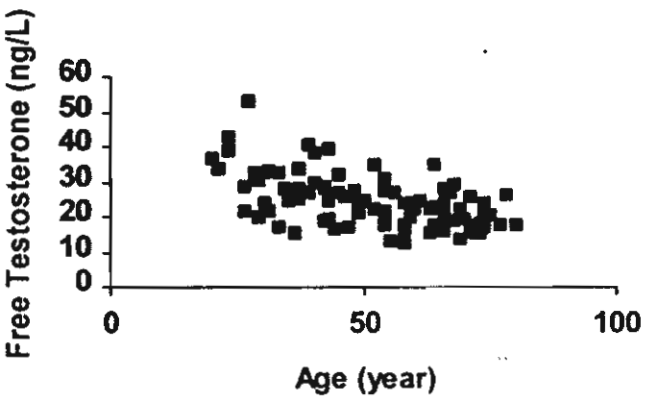


Fig. 1A Relation between advancing age and serum FT. Serum FT significantly decreases with increasing age ($r = -0.58$, $P < 0.0001$).

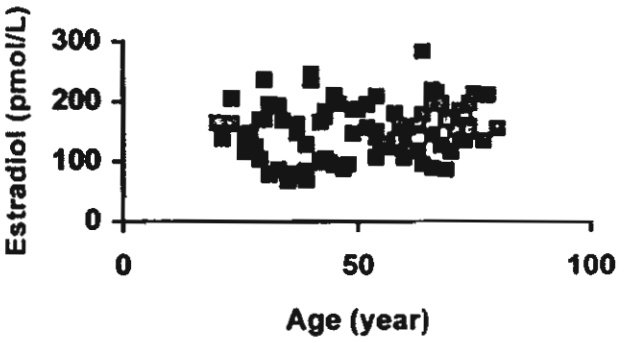


Fig. 1B Relation between advancing age and serum E2. Serum E2 did not change significantly with age.

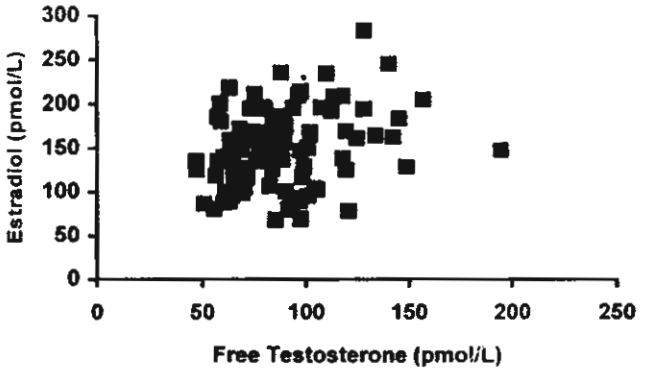


Fig. 2 Relation between serum FT and E2. Serum E2 positively correlated with serum FT ($r = 0.28$, $P < 0.05$)

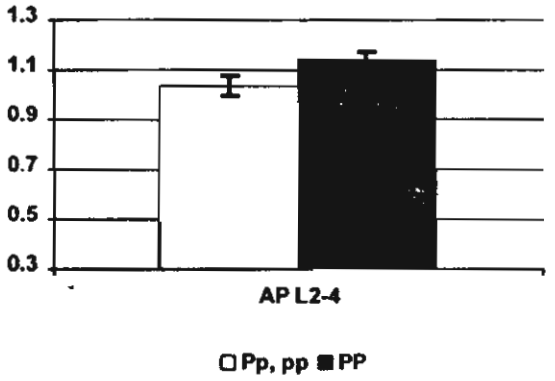


Fig. 3 Effect of ER gene polymorphism on anteroposterior L2-4 BMD. Those with the p allele had significantly lower BMD than those without.

| BMD | FT | E2 | Age | Body Weight | Fat Mass | Fat free Mass | Calcium Intake |
|---|---|---|---|---|---|---|---|
| AP L2-4 | 0.08 | 0.29[a] | 0.02 | 0.46[c] | 0.29[b] | 0.47[c] | -.0.01 |
| Lateral L2-4 | 0.17 | 0.21 | -0.39[c] | 0.48[c] | 0.26[a] | 0.52[c] | 0.02 |
| Femoral neck | 0.29[a] | 0.25[a] | -0.45[c] | 0.23[a] | -0.04 | 0.37[c] | -0.03 |
| Femoral trochanter | 0 15 | 0.27[a] | -0.03 | 0.21 | -0.02 | 0.36[b] | -0.03 |
| Ward's triangle | 0 35[a] | 0.20 | -0.52[c] | 0.15 | -0.12 | 0.30[b] | 0.001 |
| Distal radius | 0 07 | 0.14 | -0.29[b] | 0.26[a] | 0.03 | 0.346[b] | 0.02 |
| Mid radius | 0 15 | 0.13 | -0.28[a] | 0.26[a] | 0.03 | 0.357[c] | 0.05 |

Table 1 Correlation coefficients of the relations between BMD at various skeletal sites and related factors from univariate analysis.
[a] P < 0 05, [b] P < 0.01, [c] P < 0 001

| BMD | FT | E2 | Age | Body Weight | Fat Mass | Fat free Mass | Calcium Intake |
|---|---|---|---|---|---|---|---|
| AP L2-4 | NS | 0.21[a] | NS | NS | NS | 0.44[c] | NS |
| Lateral L2-4 | NS | NS | -0.30[b] | NS | NS | 0.45[c] | NS |
| Femoral neck | NS | 0.26[b] | -0.41[c] | NS | NS | 0.24[a] | NS |
| Femoral trochanter | NS | 0.22[a] | NS | NS | NS | 0.28[a] | NS |
| Ward's triangle | NS | 0.26[b] | -0.54[c] | NS | NS | NS | NS |
| Distal radius | NS | NS | NS | NS | NS | 0.37[b] | NS |
| Mid radius | NS | NS | NS | NS | NS | 0.38[b] | NS |

Table 2 Correlation coefficients of the relations between BMD at various skeletal sites and related factors from multivariate analysis.
[a] $P < 0.05$, [b] $P < 0.01$, [c] $P < 0.001$

# THE EFFECTS OF ESTROGEN EXPOSURE ON BONE MASS IN MALE TO FEMALE TRANSSEXUALS

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Running title: Effect of estrogen on bone mineral density in male to female transsexuals

Keywords: osteoporosis, bone mineral density, estrogen, transsexual

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## Abstract

The importance of estrogen on bone mineral density (BMD) in males was suggested by reports of patients with estrogen resistance and aromatase deficiency who demonstrated osteoporosis and epiphyseal plate maturation defect despite high testosterone levels. In the present study, we examined the effects of estrogen on BMD in transsexual men.

Subjects consisted of two groups of transsexual male dancers aged 16-34 years who did not receive transsexual operations (n=28). Gr I (n=11) and Gr II (n=17) have used estrogen for 2 years or less and more than 2 years, respectively. Twenty four healthy adult males served as controls. Milk consumption, smoking history and duration of regular aerobic exercise were obtained by history taking. Breast staging and testicular volume assessment by orchidometry were done. BMD measurements were performed by DEXA. BMD data shown were adjusted for body weight (BW) and analyzed by ANCOVA . Data were presented as mean ± SEM.

Signs of feminization were present in both Gr I and Gr II, with Tanner's stage II-III breast development in subjects without breast implants (n=16) and reduced testicular volume (11.2 ± 0.9 ml, n=22). There were no significant difference in age and height among the three groups. BW, amount of milk consumption and smoking, however, were different. BMD at various sites were correlated only to BW but not to smoking and milk consumption. Using ANCOVA adjusted for BW, it was found that Gr II had significant higher BMD at L2-4 and femoral neck than controls and Gr I while no difference was found between controls and Gr I as shown in the table.

| BMD ($g/cm^2$) | Controls | Gr I | Gr II |
|---|---|---|---|
| L2-4 | 1.14 ± 0.03 | 1.09 ± 0.04 | 1.22 ± 0.03 [a,b] |
| Femoral Neck | 1.01 ± 0.03 | 0.96 ± 0.04 | 1.10 ± 0.03[a,b] |

[a] $P < 05$ compared to controls. [b] $P < 0.01$ compared to Gr I

We concluded that estrogen exposure for more than 2 years in transsexual men results in an increase in bone mineral density despite signs of feminization. This supports the previous findings that estrogen may be more important than testosterone in maintaining bone mass in males.

Gonadal hormones have an important impact on bone physiology. They participate in the sexual dimorphism of skeleton, play a role in maintenance of mineral homeostasis during reproduction and are essential to maintaining bone balance in adults (1). Estrogen deficient state in postmenopausal women leads to excessive bone loss and osteoporosis. Long-term estrogen treatment reduces the incidence of fractures of the vertebrae, distal forearm and hip by about 50%. In similarity, male hypogonadism is associated with low bone mineral density that improves after testosterone therapy (2, 3).

Recent clinical observations have suggested the importance of estrogen on bone in males. Patients with complete androgen insensitiivity experienced spontaneous pubertal growth, which indicates that estrogen alone is sufficient for normal pubertal skeletal growth (4). In contrary, an estrogen resistant male patient had a severely undermineralized skeleton with biochemical evidence of increased bone resorption despite sufficient level of testosterone(5). Moreover, delayed epiphyseal closure and osteopenia are features of aromatase deficient subjects (6), and estrogen but not testosterone treatment resulted in complete epiphyseal closure and increased spinal bone mineral density (7).

The above data led to a speculation that estrogen plays a significant and probably more important role than androgen on male bone physiology such that estrogen deficient state results in low bone mass. It is unclear, nevertheless, whether estrogen supplement would increase bone mass in males without estrogen deficiency. It is thus the purpose of the present study to examine the impact of estrogen exposure on bone mass in male to female transsexuals.

## Materials and Methods

Twenty eight male to female transsexual dancers were recruited from an entertainment company in Cholburi, Thailand. None of the subjects have significant medical history or risk factors for osteoporosis such as hyperparathyroidism, thyroid disorders or glucocorticoid usage. None have received transsexual operations. Twenty four healthy men recruited from the Bangkok area, Thailand served as a control group.

All subjects gave informed consent and history of estrogen exposure including form, dosage and duration were obtained. When there were discontinuation of estrogen used, the exposure time was added up and counted for the total duration of exposure. Subjects were then divided in three groups; group I had duration of estrogen exposure $\leq$ 2 years, group II > 2 years and the control group. Additional history was taken from all subjects regarding the amount of smoking in pack-year, duration of regular aerobic exercise and milk consumption. Testicular size was assessed by orchidometry and breast was graded according to Tanner's staging.

Bone mineral density (BMD) were measured at L2-4, femoral neck, femoral trochanter, Ward's triangle and total femur by dual-energy x-ray absorptiometry (Lunar Expert XL, Lunar Corp., U.S.A.). Serum free testosterone levels were measured by radioimmunoassay (Diagnostic Product Corp., U.S.A.)

Data were presented as mean $\pm$ SEM. One-way analaysis of variance was used to assess the difference among treatment groups. Shown BMD values were adjusted for body weight and analyses of the difference in BMD after adjusted for the covariate were performed by analysis of covariance.

## Results

There were 11 subjects in group I and 17 in group II. Baseline characteristics of all subjects are shown in Table 1. No significant differences were found in age, height and amount of exercise among three groups. However, control subjects have higher body weight and amount of milk consumption than gr I and gr II. Gr II also smoked more than control group.

Within the male to female transsexual groups, gr I had mean duration of estrogen exposure of 13.9 months and gr II 58.8 months. The two groups were at a similar age at the start of estrogen usage. Signs of feminization were apparent. Mean testicular volume was found to be reduced. Gynecomastia equivalent to tanner's stage II-III were presented in all subjects without breast implants (n =14)

Forms and dosages of estrogen used in transsexuals are shown in Table 2. The dosages were mostly in pharmacological rather than physiological range. Each subject usually has used more than one form of estrogen and some have used two or three simultaneously.

Using Pearson's correlation analyses, it was found that body weight was significantly associated with BMD at various skeletal sites while milk consumption and smoking were not related to BMD as shown in Table 3. Subsequent analyses for the difference in BMD were thus performed by analyses of covariance adjusted for the difference in body weight. Adjusted BMD at various skeletal site were shown in Table 4.There were no significant differences in BMD at all sites between group 1 and controls. Group 2 had significantly higher BMD than group I at all skeletal sites while BMD were higher compared to controls at all sites except Ward's triangle. It is of note that group 1 had lower BMD than controls at AP L2-4, femoral trochanter and total femur.

## Discussion

Male to female transsexual model gave us an exellent opportunity to learn about the effects of estrogen in male. In our study, we found that the use of estrogen in a pharmacological dosage in this group led to a feminine phenotype and reduced testicular volume. Despite these findings, subjects with estrogen exposure for more than 2 years are able to maintain their BMDs at the level comparable to normal men. This findings together with others suggest that estrogen is more important than once thought in the maintenance of male skeletal health.

Sex steroids have an important impact on bone physiology. In men, the decline in serum free testosterone occurs with aging and is associated with the age-related decline in BMD (8). Male patients with various forms of hypogonadism have lower BMD and is significantly improved after restoration of eugonodal state, usually by testosterone administration. In women, estrogen, by acting through receptors in osteoblasts and various cytokines, results in changes of bone remodeling and estrogen therapy prevents postmenopausal osteoporosis and reduces fracture rate. Recently, there has been a focus on estrogen effects on bone in male. Experimental data in animals consistently showed the positive effects of estrogen on male bone. Orchidectomized male rats experienced a significant loss of cortical bone density and cancellous bone volume which was gained after estrogen treatment (9). Male Japanese quail treated with estrogen showed increase in TRAP activity and medullary bone

formation (10). In human, treatment with intramuscular estrogens in pateints with prostate cancer reduced bone turnover and maintained BMD (11) while orchidectomy resulted in an opposite pattern.Iliac bone biopsy done in estrogen-exposed male to female transsexuals showed suppressed bone turnover that was not associated with bone loss (12). Van Kesteren et al found that male to female transsexuals, when treated with one year of estrogen, had decreased bone turnover and increase in BMD (13). Our results are in an agreement with the aforementioned findings and suggests that the positive effect of estrogen on male skeletal health may persist up to as long as 8 years of estrogen exposure.

The results of this study may be affected by the different forms, dosages and irregularity of estrogen used in each transsexual subjects. However, the effect of estrogen on bone mass is likely to be cumulative and the influence of the total exposure time should not be distorted by the disruptions of usage. Twenty two of our subjects are current users and four of the rest six have used estrogen for at least 6 months in the last year. Overall, this is not likely to greatly affect the results.

In conclusion, we have demonstrated that estrogen has a positive effect on bone mineral density in transsexual male. Estrogen exposure for more than two years is associated with an increase in bone density despite low serum free testosterone levels. This supports the previuos findings that estrogen is an important factor in maintaining bone mineral density in male.

| | Gr I (n= 11) | Gr II (n=17) | Control (n=24) |
|---|---|---|---|
| age (yr) | 21.2 ± 1.12 | 24.12 ± 0.83 | 24.17 ± 0.8 |
| (range) | (16-30) | (20-32) | (19-34) |
| height (cm) | 166.34 ± 1.44 | 166.61 ± 1.38 | 167.19 ± 1.12 |
| weight (kg) | 52.77 ± 2.10 | 55.99 ± 1.72 | 61.17 ± 1.50 [a] |
| Exercise (yr) | 1.17 ± 0.54 | 4.27 ± 1.12 | 4.43 ± 0.62 |
| milk (glasses/wk) | 0.72 ± 0.22 | 0.47 ± 0.12 | 4.25 ± 0.87 [b] |
| Smoking (pack-yr) | 2.27 ± 1.6 | 4.03 ± 0.92 [c] | 0.00 |
| Estrogen exposed | 13.91 ± 2.27 | 58.82 ± 5.04 [d] | |
| Duration (months) | (1.0 - 24.0) | (28.0 - 96.0) | |
| age starting estrogen [e] | 20.03 ± 1.07 | 19.14 ± 0.98 | |
| Testicular size(ml) | 10.13 ± 1.81 | 11.82 ± 1.05 | |

[a] significantly higher than gr I and gr II ; $P < 0.05$
[b] significantly higher than gr I and gr II ; $P < 0.05$
[c] significantly higher than control group ; $P < 0.05$
[d] significantly higher than gr I ; $P < 0.05$
[e] age at onset of estrogen usage

Table 1 Baseline chracteristics of subjects

| Forms | Types | Dose |
|---|---|---|
| Injection | estradiol valerate 10 mg / ampule | 1-4 /month |
| Contraceptive Pills | mestranol 0.05 mg + noretisterone 1 mg<br>ethinyl estradiol 0.03 mg + levonogestrel 0.15 mg<br>ethinyl estradiol 0.035 mg + cyproterone acetate 2 mg<br>Triphasic pills<br>ethinyl estradiol 0.03 mg + levonogestrel 0.05 mg<br>ethinyl estradiol 0.04 mg + levonogestrel 0.075 mg<br>ethinyl estradiol 0.03 mg + levonogestrel 0.125 mg | 1-4 tabs/day |
| Oral estrogen | conjugated estrogen 1.25 mg | 1-2 tabs/day |

Table 2 Forms and dosgaes of estrogen used

| BMD | Body Weight | Milk Consumption | Smoking |
|---|---|---|---|
| AP L2-4 | 0.48* | NS | NS |
| Femoral Neck | 0.53* | NS | NS |
| Femoral Trochanter | 0.41* | NS | NS |
| Ward's Triangle | 0.47* | NS | NS |
| Total Femur | 0.50* | NS | NS |

*P < 0.01

Table 3 Correlation between BMD at various skeletal sites and factors which may affect bone mass.

| BMD | Control | Group 1 | Group 2 |
|---|---|---|---|
| AP L2-4 | 1.14 ± 0.03 | 1.08 ± 0.03 [c] | 1.22 ± 0.03 [a, b] |
| Femoral Neck | 1.01 ± 0.03 | 0.95 ± 0.03 | 1.10 ± 0.03 [a, b] |
| Femoral Trochanter | 0.83 ± 0.03 | 0.70 ± 0.04 [c] | 0.84 ± 0.03 [a, b] |
| Ward's Triangle | 0.90 ± 0.03 | 0.86 ± 0.05 | 1.00 ± 0.04 [d] |
| Total Femur | 1.07 ± 0.03 | 0.96 ± 0.05 [c] | 1.11 ± 0.03 [a] |

[a] P < 0.01 compared to control, [b] P < 0.05 compared to Group 1, [c] P < 0.05 compared to control, [d] P < 0.05 compared to group 1.

Table 4 Comparisons of BMD in control, transsexuals on estrogen for 2 years or less (Group 1) and transsexuals on estrogen for more than 2 years (Group 2).

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