รายงานวิจัยฉบับสมบูรณ์

โครงการ Mutational analysis ของ estrogen receptor gene และบทบาทของ estrogen ร่วมกับ estrogen receptor gene locus ต่อโรคกระดูกพรุน, glucose และ lipid metabolism ในชาย

โดย

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

กิตติกรรมประกาศ

คณะผู้วิจัยขอขอบพระคุณสำนักงานกองทุนสนับสนุนการวิจัย และคณะแพทยศาตสร์ โรงพยาบาลรามาธิบดี มหาวิทยามหิดล ที่ได้ให้การสนับสนุนจนงานวิจัยนี้สำเร็จลุล่วงด้วยดี

รหัสโครงการ RSA408011

ชื่อโครงการ Mutational analysis ของ estrogen receptor gene และบทบาทของ estrogen ร่วมกับ estrogen receptor gene locus ต่อโรคกระดูกพรุน, glucose และ lipid metabolism ในชาย

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การศึกษานี้มีวัตถุประสงค์เพื่อหา single nucleotide polymorphisms (SNPs) บน coding และ promotor region ของ estrogen receptor-α (ERα) gene และศึกษาความสัมพันธ์ของ SNPs เหล่านี้ต่อความเสี่ยงที่จะเกิดโรคกระดูกพรุนและการตอบสนองต่อการรักษาด้วย estrogen นอกจากนี้ ยังมีวัตถุประสงค์เพื่อศึกษบทบาทของ estrogen และ SNPs เหล่านี้ ในการเกิดโรคกระดูกพรุนในชาย

จากการหาลำดับเบสของ ERα gene พบว่ามี T262C และ G2014A SNPs บน exon 1 และ 8 ตามลำดับ หลังการรักษาสตรีวัยหมดประจำเดือนจำนวน 96 รายด้วย 0.3 mg หรือ 0.625 mg ของ conjugated equine estrogen (CEE) เป็นเวลา 2 ปี พบว่าความหนาแน่นของกระดูกบริเวณกระดูกสันหลังเพิ่มขึ้นโดยไม่มีความสัมพันธ์กับ T292C SNP อย่างไรก็ตาม ผู้ป่วยที่ homozygous สำหรับ 262C allele เท่านั้นที่มีความหนาแน่นของกระดูกบริเวณ femoral neck เพิ่มขึ้น (+5.9 ± 1.4%, mean ± SEM, $P < 0.0001$) ในสตรีวัยหมดประจำเดือนอายุมากกว่า 55 ปี 228 ราย ซึ่งเป็นโรคกระดูกพรุน 106 ราย และไม่เป็นโรคกระดูกพรุน 122 ราย พบว่า G2014A SNP เป็นปัจจัยเสี่ยงของการเป็นโรคกระดูกพรุน (odds ratio 2.7 per A allele, 95% CI 1.49-4.76) โดยไม่ขึ้นกับน้ำหนักตัว (odds ratio 0.93 per kg, 95% CI 0.89-0.96) และระยะเวลาหลังหมดประจำเดือน (odds ratio 1.12 per year, 95% CI 1.08-1.19) ส่วนในชายอายุอย่างน้อย 60 ปี จำนวน 98 ราย ซึ่งมีผู้ที่เป็นโรคกระดูกพรุน 18 รายพบว่าระดับ estradiol ไม่แตกต่างกันระหว่างผู้ที่เป็นและไม่เป็นโรคกระดูกพรุน (90.6 ± 12.7 vs. 88.7 ± 5.0 pmol/L) การกระจายตัวของ T262C และ G2014A genotype ไม่มีว่าแตกต่างกันระหว่างผู้ที่มีหรือไม่มีโรคกระดูกพรุน มีเพียงน้ำหนักตัวเท่านั้น (OR 0.86, 95% CI 0.78-0.94) ที่มีความสัมพันธ์กับการเป็นโรคกระดูกพรุนในเพศชาย อย่างไรก็ตาม ผู้ป่วยชายที่มีภาวะ hypogonadism 9 ราย หลังได้ 0.3 mg CEE พบว่ามีระดับ CTX ในเลือดซึ่งเป็นดัชนีของการสลายกระดูกลดลง นอกจากนี้ ยังพบว่าในผู้ป่วยเหล่านี้มี glucose effectiveness เพิ่มขึ้นโดยไม่พบการเปลี่ยนแปลงของ insulin sensitivity หรือระดับไขมันในเลือดหลังได้รับ CEE

โดยสรุป คณะผู้วิจัยได้พบ SNPs บน ERα gene ซึ่งน่าจะมีความสำคัญในด้าน pharmacogenetics และในด้านการทำนายโรคกระดูกพรุนในสตรีวัยหมดประจำเดือน ผลการศึกษาดังกล่าวยังต้องการการศึกษาเพิ่มเติมเพื่อยืนยันและบอกกลไกการทำงานที่แน่ชัด การศึกษานี้ในผู้ป่วยชายชี้แนะว่าโรคกระดูกพรุนในชายอาจมีปัจจัยทางพันธุกรรมและพยาธิกำเนิดแตกต่างจากโรคกระดูกพรุนในสตรีวัยหมดประจำเดือน

คำหลัก estrogen, estrogen receptor gene, โรคกระดูกพรุน

Project Code: RSA408011

Project Title: Mutational analysis of estrogen receptor gene and the role of estrogen together with estrogen receptor gene locus in male osteoporosis, glucose and lipid metabolism

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Project Period: 1 December 1997-30 November 2000

In the present study we searched for single nucleotide polymorphisms (SNPs) in the coding and promotor regions of estrogen receptor-α (ERα) gene and assessed the relations of these SNPs with the risk of postmenopausal osteoporosis and skeletal responsiveness to estrogen. The roles of estrogen and these SNPs in male osteoporosis were also assessed.

Direct sequencing of the coding and promotor regions of ERα gene revealed a T262C and a G2014A SNP in exon 1 and 8, respectively. After treating 96 postmenopausal with 0.3 mg or 0.625 mg conjugated equine estrogen (CEE) for 2 years, vertebral bone mineral density (BMD) increased regardless of the T262C genotype. However, with regard to femoral neck BMD, only those homozygous for the 262C allele had an increase in femoral BMD (+5.9 $\pm$ 1.4%, mean $\pm$ SEM, $P < 0.0001$). In 228 postmenopausal women aged more than 55 years with vertebral or femoral osteoporosis (n = 106) and without osteoporosis (n = 122), the G2014A polymorphism was related to the presence of osteoporosis (odds ratio 2.7 per A allele, 95% CI 1.49-4.76) independently of body weight (odds ratio 0.93 per kg, 95% CI 0.89-0.96) and years since menopause (odds ratio 1.12 per year, 95% CI 1.08-1.19).

In 98 males aged 60 years or more, of whom 18 had vertebral or femoral osteoporosis, no significant difference in circulating estradiol was detected (90.6 $\pm$ 12.7 vs. 88.7 $\pm$ 5.0 pmol/L). The genotype distributions of the T262C SNP in exon 1 and G2014A SNP in exon 8 did not differ in subjects with and without osteoporosis. Only body weight (OR 0.86, 95% CI 0.78-0.94) was independently associated with osteoporosis. However, administration of 0.3 mg CEE to 9 hypogonadal males caused a decrease in serum CTX, a marker of bone resorption. Glucose effectiveness increased after CEE whereas no effect on insulin sensitivity or serum lipid concentrations was detected.

In conclusions, we have identify SNPs in ERα gene which is likely to be significant pharmacogenetically and prosnostically in postemenopausal osteoporosis. Further studies is needed to confirm the findings and delineate the underlying mechanisms. As compared to females, our finding suggests that the disorder in males may be genetically and pathophysiologically different.

Keywords: estrogen, estrogen receptor gene, osteoporosis

The objectives of the present study were

1. Assess the relation between the PvuII single nucleotide polymorphism (SNP) in intron 1 of estrogen-receptor-α (ER α) gene and skeletal responsiveness to estrogen.
2. Identify SNPs in exons of ERα gene which may be in linkage disequilibrium with the intronic PvuII SNP and their roles in postmenopausal osteoporosis.
3. Investigate the effect of exogenous estrogen on bone, lipid and glucose metabolism in males.
4. Assess the role of endogenous estrogen together with SNPs in ERα gene as risk factors for idiopathic osteoporosis in males.

The study was conducted during December 1997 and November 2000. The results of which are presented in 5 separate reports namely:

1. Estrogen-receptor-α Gene polymorphism affects response in bone mineral density to estrogen in postmenopausal women
2. Association of a T262C transition in exon 1 of estrogen-receptor-α gene with skeletal responsiveness to estrogen in postmenopausal women
3. Association of a G2014A transition in exon 8 of estrogen-receptor-α gene with postmenopausal osteoporosis
4. Effect of estrogen replacement on glucose sensitivity, serum lipids and bone markers in hypogonadal males
5. Circulating estradiol and estorgen-receptor-gene polymorphisms in elderly men with idiopathic osteoporosis

ESTROGEN-RECEPTOR-α GENE POLYMORPHISM AFFECTS RESPONSE IN BONE MINERAL DENSITY TO ESTROGEN IN POSTMENOPAUSAL WOMEN

Osteoporosis is partly genetically determined. A number of candidate genes for osteoporosis have been examined. For examples, it was found that subjects with the bb vitamin D receptor (VDR) genotype have higher bone mineral density (BMD) compared to other genotypes (1). However, a number of subsequent studies were unable to confirm such association (2). Nevertheless, the VDR polymorphism has been shown to be associated with functional differences in terms of intestinal calcium absorption and suppression of parathyroid hormone levels after treatment with calcitriol (3). Besides VDR polymorphisms, estrogen-receptor-α (ERα) gene polymorphisms have also been associated with BMD in some (4-6) but not all studies (7, 8). The ERα gene polymorphisms thus far studied reside in introns and their functional significance is unclear. In fact, a previous study could not demonstrate the association between skeletal responsiveness to estrogen and ERα genotype (8). However, the doses of estrogen used was not clearly stated. If ERα gene polymorphisms do affect skeletal response, it is likely that such differences may be more readily elicited at lower doses of estrogen. It is therefore the purpose of the present study to prospectively assess the difference in skeletal response to estrogen replacement in relation to ERα genotypes and doses of estrogen in postmenopausal women.

Materials and Methods

Subjects

Subjects consisted of 124 postmenopausal women; 63 were less than 6 years postmenopausal (group 1) while 61 were more than 10 years postmenopausal with osteoporosis as defined by lumbar or femoral neck BMD lower than –2.5 standard deviation of the mean of Thai young women (group 2). All subjects did not smoke nor drink and did not engage in regular strenuous exercise. The subjects were recruited by flyers or direct contact. 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.

Medications

Subjects were randomly allocated to one of the two treatments, 0.3 or 0.625 mg of conjugated equine estrogen (CEE). Subjects in group 1 with an intact uterus were also given 5 mg of medrogestone acetate from day 1 to 12 each month while those in group 2 took the drug daily. Each subject also took 750 mg elemental calcium supplementation in the form of calcium capsules daily. The compliance to medication was confirmed by counting the remaining tablets at each visit.

Bone mineral densitometry

Bone mineral densities (BMD) were measured by dual-energy X-ray absorptiometry (Lunar Expert XL, Lunar Corp., U.S.A.). Daily calibration and quality control were done regularly according to the manufacturer's recommendation. BMD at anteroposterior L2-4 and femoral neck were measured in each subject. In vivo coefficients of variation for these sites were 1.2% and 1.6%, respectively.

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 osteocalcin (OC) was determined by enzyme immunoassay (Metra Biosystems, U.S.A.). Twenty-four-hour urine was collected in each subject. Urinary calcium and creatinine were determined by standard methods. Urinary deoxypyridinoline crosslink (DPD) were assessed by enzyme immunoassay (Metra Biosystems, U.S.A.). The intraassay coefficients of variation for OC and DPD were 12.9% and 5.2%, respectively.

ERα genotyping

Genomic DNA was extracted from peripheral leukocytes by phenol/chloroform extraction. DNA sequence flanking the polymorphic site in intron 1 of ERα gene was amplified by polymerase chain reaction with the following primers: forward, 5'CTGCCACCCTATCTGTATCTTTTCCTATTCTCC; and reverse, 5'TCTTTCTCTGCCACCCTGGCGTCGATTATCTGA (4). The 50 μL final reaction contained 0.1 μg DNA, 1 unit of Taq polymerase, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1 mM $MgCl_2$, 200 μM each of the four deoxyribonucleotides and 0.4 μM each of the primers. The reaction was run for 30 cycles with denaturation at 94 °C for 30 seconds, annealing at 61 °C for 40 seconds and extension at 72 °C for 90 seconds. The final PCR product was then digested with *Pvu II* restriction endonuclease. The digested material was resolved on 1.4% agarose gel with ethidium bromide staining. Given the repeatability of the assay (100% in 20 samples), the assay was run once, unless ambiguous, without an internal control for each sample. The results were read without the knowledge of BMD and treatment data. Capital P represents the absence of the restriction site while small p indicates the presence of the restriction site.

Statistical analyses

Data were expressed as mean ± SEM. Two-way ANOVA was used to assess the combined effects of the dose of CEE and ERα genotype on the changes in BMD. Differences in independent variables were analyzed by one-way ANOVA or ANCOVA. Changes in BMD and biochemical markers of bone turnover were assessed by paired Student's t test.

Results

The genotype distribution was as follows: pp 40 (32.3%), Pp 63 (50.8%) and PP 21 (16.9%) which conformed to the Hardy-Weiberg equilibrium. As shown in Table 1 there was no difference in age, years since menopause, body weight and biochemical markers of bone turnover among subjects with different genotypes. Although BMD at L2-4 and femoral neck tended to be lower in subjects with PP genotype, this did not reach statistical significance. Likewise, no difference in baseline characteristics in relation to the doses of CEE was found.

When assessing the difference in the change in BMD after estrogen in relation to doses and genotype, it was found by two-way ANOVA that there is a significant effect of the dosage of CEE ($p < 0.05$) but the ERα genotype was of no effect ($p = 0.17$). However, there was a two-way interaction between genotype and the dosage of estrogen ($p < 0.05$). Therefore the analyses were performed separately for each dose of estrogen. As shown in Table 2, 0.3 mg CEE did not significantly affect vertebral BMD in women with the pp genotype. In contrast, subjects with Pp or PP genotypes had increased vertebral BMD after 1-year treatment with 0.3 mg CEE. There was a significant correlation between ERα genotypes and the proportion of subjects who had 3% or more increase in vertebral BMD after treatment (Spearmen's rho = 0.26, $p < 0.05$). The proportion

increased from 55% (11/20) in pp genotypes to 64.7% (22/34) and 92.3% (12/13) in Pp and PP genotypes, respectively. No change in BMD was demonstrated at the femoral neck regardless of genotype. In contrast to the findings with 0.3 mg CEE, 0.625 mg CEE caused an approximately 8% increase in L2-4 BMD regardless of genotype. As with 0.3 mg CEE, no significant change was demonstrated at the femoral neck.

After correcting for age and the number of years since menopause using analysis of covariance, subjects on 0.3 mg CEE with the P allele still had significantly higher increase in L2-4 BMD compared to those without the P allele ($p < 0.05$). No difference in the change at femoral neck was found. Neither the changes in verterbral nor femoral BMD were different among subjects on 0.625 mg CEE with different genotypes (Table 3). In terms of biochemical markers of bone turnover after adjusting for age and years since menopause, there was no difference in the decrease of serum OC or urinary DPD in relation to the dose of estrogen or genotype.

Discussion

Pvu II and *Xba I* RFLPs in intron 1 of ERα gene have been found in some association studies, but not all, to be related to bone mass. Moreover, the direction of change in bone mass in relation to the *Pvu II* RFLP appeared to be different among studies. These conflicting results make the contribution of ERα gene polymorphisms to osteoporosis unclear. Moreover, the polymorphisms studied thus far are located in an intron and are thus of uncertain functional significance. In the present study, we demonstrated that the intronic *Pvu II* RFLP was related to response in vertebral BMD and the relation was evident only with low-dose CEE. The genotype distribution in the present study was similar to previous studies in Thais (6) and also to that in Caucasians (7). The association between the ERα gene polymorphism and skeletal response suggests that the intronic polymorphism possesses functional significance. However, the effect is minor as suggested by the inability of higher dose of CEE to elicit the difference in response. Confounded by other genetic and environmental factors, the contribution of ERα polymorphism to osteoporosis may be difficult to be identified by an association study. Intervention with relevant agents which act through the protein of interest may thus be helpful in investigating the importance of candidate genes in the pathogenesis of osteoporosis. Our finding is consistent with a recent study in Caucasian elderly women which suggested a relationship between changes in BMD after estrogen replacement and vitamin D receptor and ERα genotypes (9). However, it is different from a study in Korean patients where no association between skeletal response and ERα gene polymorphism was found (8). Subjects were not separately analyzed based on the doses of estrogen in that study. Doses of medication may need to be considered with respect to the effect of genetic polymorphism on the responses to the treatment.

Single nucleotide polymorphisms (SNP) occur throughout the human genome (10). Although the function of these nucleotide changes is unclear, they can serve as genetic markers for studying the genetic basis of diseases. Recently, it has been demonstrated that SNP in certain genes may affect drug or dietary responses (11, 12). In regard to estrogen replacement therapy, 0.625 mg of CEE is generally considered to be the optimal dose to prevent bone loss in postmenopausal women. Recently, however, residual endogenous estrogen in postmenopausal women was demonstrated to be related to fracture risk (13) and the reduction in endogenous estrogen by aromatase inhibitor can increase bone turnover (14). These findings combined suggest that lower doses of estrogen may be effective in the preservation of bone mass in postmenopausal women. This is in keeping with previous studies that in the presence of calcium supplementation, lower doses of estrogen are protective for bone (15) without stimulating the

endometrium (16). The finding in the present study that the response to low-dose estrogen is related to ERα genotype raises the possibility that lower doses of estrogen may not be equally effective in all individuals. One of the limitation of our study is the relatively small number of subjects. Power calculations for the change in vertebral BMD in subjects with the pp genotype on low-dose CEE revealed that our sample was adequate to detect a 5% change with 80% power at 95% confidence. However, when compared to the vertebral response in subjects on the higher dose, the extent of change in subjects on 0.3 mg CEE was significantly lower. If the present finding is confirmed in other studies, clinical trials looking at the skeletal effect of low-dose estrogen should also take ERα genotype into consideration since ERα genotyping may help identify women who would be most likely to respond to low-dose estrogen. Whether lower doses of estrogen in those with a favorable skeletal response will possess less side effects in terms of breast and endometrium stimulation is unknown. Moreover, whether detecting ERα genotype will help in identifying postmenopausal women whose serum lipid levels would respond favorably to low-dose estrogen remains to be determined.

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	Genotype				Dose of CEE		
	pp (n = 40)	Pp (n = 63)	PP (n = 21)	p	0.3 mg (n = 67)	0.625 mg (n = 57)	p
Age (year)	59.9 ± 1.2	57.9 ± 1.0	59.7 ± 2.0	NS	58.4 ± 1.0	59.4 ± 1.1	NS
Years sine menopause (year)	13.1 ± 1.7	8.6 ± 0.9	12.7 ± 2.4	NS	10.9 ± 1.2	11.1 ± 1.2	NS
Body weight (kg)	57.7 ± 1.4	56.7 ± 1.2	55.1 ± 3.2	NS	56.4 ± 1.5	57.2 ± 1.1	NS
L2-4 BMD(g/cm^2)	0.94 ± 0.03	0.94 ± 0.02	0.87 ± 0.04	NS	0.92 ± 0.02	0.94 ± 0.02	NS
Femoral Neck BMD(g/cm^2)	0.80 ± 0.03	0.79 ± 0.02	0.73 ± 0.03	NS	0.78 ± 0.02	0.78 ± 0.02	NS
OC (ng/ml)	9.3 ± 1.0	11.1 ± 0.8	10.4 ± 1.4	NS	9.6 ± 0.6	8.9 ± 0.6	NS
DPD (nmole/mmole creatinine)	4.3 ± 0.2	4.8 ± 0.2	4.2 ± 0.4	NS	4.3 ± 0.2	4.9 ± 0.3	NS

Table 1 Baseline characteristics (mean ± SEM) of subjects in relation to ERα genotype and the dose of CEE.

0.3 mg CEE					0.625 mg CEE				
	L2-4 BMD		Femoral Neck BMD			L2-4 BMD		Femoral Neck BMD	
Genotype (n)	% Change	P	% Change	p	Genotype (n)	% Change	p	% Change	p
pp (20)	2.3 ± 2.1	NS	1.6 ± 1.0	NS	pp (20)	8.7 ± 1.6	< 0.001	3.5 ± 2.1	NS
Pp (34)	7.6 ± 1.5	< 0.001	2.0 ± 1.2	NS	Pp (29)	7.8 ± 1.4	< 0.001	2.1 ± 2.0	NS
PP (13)	6.9 ± 1.0	< 0.001	2.0 ± 1.2	NS	PP (8)	7.7 ± 2.6	< 0.001	-0.1 ± 2.1	NS

Table 2 Changes in L2-4 and femoral neck BMD (mean ± SEM) after 1-year of 0.3 and 0.625 mg of CEE. The p values of less than 0.05 indicate significant changes compared to baseline values.

	0.3 mgCEE			0.625 mg CEE		
	pp genotype (n = 20)	**Pp or PP genotypes (n = 47)**	**p**	**pp genotype (n = 20)**	**Pp or PP genotypes (n = 37)**	**p**
Change in L2-4 BMD (%)	2.8 ± 1.8	7.2 ± 1.2	< 0.05	7.8 ± 1.8	8.2 ± 1.3	NS
Chane in Femoral neck BMD (%)	1.6 ± 1.3	2.0 ± 0.9	NS	1.6 ± 1.6	2.8 ± 1.2	NS
Change in OC (%)	-11.0 ± 5.5	-23.2 ± 3.5	NS	-6.5 ± 9.7	-18.6 ± 6.8	NS
Change in DPD (%)	-14.3 ± 8.0	-13.6 ± 5.6	NS	-26.9 ± 7.7	-22.6 ± 6.0	NS

Table 3 Change in L2-4, femoral neck BMD and biochemical markers of bone turnover (mean ± SEM) in relation to the doses of estrogen and ERα genotypes. Values shown were adjusted for effect of age and years since menopause.

ASSOCIATION OF A T262C TRANSITION IN EXON 1 OF ESTROGEN-RECEPTOR-α GENE WITH SKELETAL RESPONSIVENESS TO ESTROGEN IN POSTMENOPAUSAL WOMEN

It is well established that postmenopausal osteporosis is partly genetically determined. A number of candidate genes such as the vitamin D receptor gene, estrogen-receptor -α gene (ERα) and type 1 collagen gene have been investigated in association studies to elucidate the genetic nature of osteoporosis. The results from studies in various populations are still in dispute suggesting the complex nature of the genetics of postmenopausal osteoporosis.

As for ERα gene, a number of studies have found an association between ERα gene polymorphisms and bone mass while no association was detected in others (1-9) The markers of ERα gene studied thus far mostly involve a PvuII and a XbaI restriction fragment length polymorphisms which reside in intron 1 of ERα gene. The functional significance of these polymorphisms is not entirely clear and it is likely that these intronic polymorphisms may be in linkage disequilibrium with other nucleotide changes in the nearby exons of ERα gene or its 5' regulatory sequence. It is the purpose of the present study to search for nucleotide changes in the exon 1 and regulatory sequences of ERα gene, the nature of their linkages to the previously reported PvuII polymorphism and their association with the response in bone mineral density (BMD) to estrogen.

Materials and Methods

Subjects

All subjects were recruited by direct contact or advertising through flyers. Informed consent was obtained from each subject 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.

Mutational analysis of the promotor region and exon 1 or ERα gene

Mutational analysis was performed in 19 postmenopausal women with lumbar or femoral neck osteoporosis as defined by the T-score of bone mineral density (BMD) below –2.5. All were otherwise healthy and did not take medication which may affect calcium and bone metabolism.

The genomic DNA of patients was extracted from peripheral leukocytes. Exon 1 of ERα gene was amplified by PCR with the following primers: forward, 5'-GTTTCTGAGCCTTCTGCCCTG and reverse, 5'-GCGCGGGTACCTGTAGAATG (10) The final reaction contained 0.5 μg DNA, 1 unit of Taq polymerase, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$. The reaction was run for 30 cycles with denaturation at 95 °C for 60 seconds, annealing at 67 °C for 60 seconds and extension at 72 °C for 30 seconds. DNA sequence in the promotor region including nucleotides up to position 128 upstream from the beginning of exon 1 was amplified using the following primers forward, 5'-GAGTTGTGCCTGGAGTGATG and reverse, 5'-ACCTGGAAAAAGAGCACAGC. The final reaction contained 0.5 μg DNA, 1 unit of Taq polymerase, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1 mM $MgCl_2$. The reaction was run for 30 cycles with denaturation at 95 °C for 30 seconds, annealing at 63 °C for 30 seconds and extension at 72 °C for 30 seconds. The DNA fragments were then subjected to direct dye cycle sequencing (ABI Prism 310) in the forward and backward directions using the corresponding PCR primers.

Linkage disequilibrium analysis between the intronic PvuII and T262C polymorphisms

In 129 postmenopausal women, the intronic PvuII polymorphism was assessed as previously described (2). P represents the absence of the PvuII restriction site whereas p indicates the presence of the restriction site. The T262C nucleotide change in exon 1 was genotyped by allele-specific PCR using a common primer, 5'TTGCTGCTGTCCAGGTACAC and 2 specific primers, 5'CCCTCCACACCAAAGCATCT and 5'CCTCCACACCAAAGCATCC. The final reaction contained 0.5 μg DNA, 1 unit of Taq polymerase, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1 mM $MgCl_2$. The reaction was run for 30 cycles with denaturation at 95 °C for 30 seconds, annealing at 63 °C for 30 seconds and extension at 72 °C for 30 seconds. The product was then resolved on 3 % agarose gel with ethidium bromide staining. Linkage disequilibrium was assessed by the chi-square test.

Responses in BMD after estrogen treatment in relation to ER gene polymorphism

Ninety six postmenopausal women were included in the study. Forty seven were less than 6 year postmenopausal (group 1) while 49 were more than 10 years postmenopausal with osteoporosis (group 2) as defined by lumbar or femoral neck BMD lower than –2.5 standard deviation of the mean of Thai young women.

Subjects were randomly allocated to one of the two treatments, 0.3 or 0.625 mg of conjugated equine estrogen (CEE). Subjects in group 1 with intact uterus were also given 5 mg of medrogestone acetate from day 1 to 12 each month while those in group 2 took the drug daily. Each subject also took 750 mg elemental calcium supplementation in the form of calcium capsule daily.

Bone mineral densities (BMD) were measured by dual-energy X-ray absorptiometry (Lunar Expert XL, Lunar Corp., U.S.A.). Daily calibration and quality control were done regularly according to the manufacturer's recommendation. BMD at anteroposterior L2-4 and femoral neck were measured in each subject. In vivo coefficients of variation for these sites were 1.2% and 1.6%, respectively. Restriction fragment length polymorphisms of ERα gene were determined as described above.

Changes in BMD from baseline were assessed by paired Student's t test. Analysis of covariance was performed to determine the effects of various variables on the change in BMD after treatment. Data were expressed as mean ± SEM.

Results

Direct sequencing of exon 1 and promotor region of ERα gene revealed a synonymous nucleotide substitution from T to C at position 262, 29 nucleotides from the putative start codon in 5 of 19 osteoporotic subjects. No nucleotide change was found in the promotor region. Linkage disequilibrium between T262C polymorphism and PvuII polymorphism in intron 1 of ERα gene was demonstrated in 129 postmenopausal women as shown in Table 1. The C262 allele appeared to be in linkage with the P allele of the intronic polymorphism.

As for the study regarding the relation between T262C polymorphism and the changes in BMD after CEE, there was no difference in clinical characteristics at the beginning of the study among subjects with different genotypes except for a lower femoral BMD in subjects with the T262C polymorphism (Table 2). Table 3 demonstrates the changes in vertebral and femoral BMD after treatment with 0.3 mg or 0.625 mg CEE for 2 years. In 51 postmenopausal women who was given 0.3 mg CEE, there was a significant increase in vertebral BMD ($P < 0.001$) after treatment while no change was demonstrated at the femoral neck. Similar results were demonstrated in subjects on 0.625 mg CEE. After dividing the subjects according to the presence of the T262C polymorphism, it was found that verbebral BMD increased

after 2-year administration of CEE regardless of the T262C genotype. However, with regard to femoral neck BMD, only those homozygous for the T262C polymorphism had a significant increase in femoral BMD whereas in subject heterozygous for the polymorphism femoral BMD tended to increase but did not reach statistical significance (Table 5).

Using analysis of covariance to assess the effects of the T262C polymorphism, the intronic PvuII polymorphism, doses of CEE and the corresponding baseline BMD on the changes in vertebral or femoral BMD after treatments as shown in Table 6, it was found that the change in vertebral BMD was related only to the baseline L2-4 BMD. However, the change in femoral BMD was independently related to T262C polymorphism and the baseline femoral BMD. No effect of the PvuII polymorphism or the doses of CEE was demonstrated.

Discussion

A number of candidate genes have been implicated in the determination of bone mass and the pathogenesis of osteoporosis. For examples, vitamin D receptor (VDR) gene polymorphism have been widely studied and shown to inconsistently affect bone mass (11-16) and influence intestinal calcium absorption (17-19) Other less well studied candidate genes include those coding for ERα, type 1 collagen and interleukin-1. Regarding ERα gene, almost all studies so far involve polymorphic sites in the non-coding region of the gene, namely the PvuII and XbaI polymorphism in intron 1 (2-9) and microsatellite markers upstream to the ERα gene (1). The results are still not without dispute. Despite such inconsistency, there is evidence suggesting the relation between these polymorphisms and skeletal responsiveness to estrogen (20) and it is possible that polymorphic markers studied thus far may not be directly involve in the physiology of bone mass determination. Our finding that the T262C polymorphism, which has been previously described (21), is in linkage disequilibrium with the PvuII polymorphism in intron 1 and is more related to changes in bone mass after estrogen treatment suggests that the T262C polymorphism may be more directly involve in modulating the effect of estrogen on bone and possibly on the pathogenesis of osteoporosis.

There has been evidence that tissue responsiveness to estrogen is under complex genetic control. Recently, two quantitative loci on mouse chromosomes 5 and 11 have been identified to control the phenotypic variation in uterine wet weight in response to estrogen (22). The T262C single nucleotide polymorphism at the ERα gene as shown in the present study may represent another level of genetic modulation of estrogen responsiveness. However, it is unclear how the synonymous nucleotide change influences the function of ERα. One of the possibilities is that the T262C polymorphism may affect an alternative translation initiation site. Generally the ATG codon with appropriate context nearest the 5' end of the mRNA serves as the initiation codon (23) and polymorphism of nucleotide sequence around the initiation codon has recently been described to influence the surface levels a cell adhesion receptors (24). Occasional escape from this first-ATG rule occurs. The ERα T262C polymorphism is located 29 nucleotides downstream from the putative translation initiation site in the vicinity of another ATG codon around which the context (GCATC[T/C]GGGATGG) may be appropriate for it to serve as another translation initiation site and the polymorphism may influence the favorableness of its being an alternative start codon. More studies regarding this issue are needed to be performed.

Estrogen has been widely used for the prevention and treatment of postmenopausal osteoporosis. An increase in vertebral bone mass if adequate doses of estrogen are administered and reduced vertebral fractures have been demonstrated (25). On the other hand, the effect of estrogen on bone mass at the

femoral neck is more limited (26-29). Likewise, only increase in vertebral but not femoral fractures was demonstrated with selective estrogen receptor modulator (30). The less femoral responsiveness regarding BMD after estrogen replacement has been attributed largely to less proportion of trabecular bone at this skeletal site. The finding in our study that BMD at the femoral neck responded more favorably to estrogen in those with the T262C polymorphism may partly explain the inconsistency among studies in different populations. Of all the antiresorptive agents available for osteoporosis, only bisphosphonates have been consistently demonstrated to be able to increase femoral bone mass for postmenopausal women with femoral osteoporosis (31, 32) and bisphosphonates rather than estrogen may be more favorable for the prevention of hip fractures. Stratifying postmenopausal women with femoral osteoporosis according to ERα T262C genotype before treatment with estrogen may be helpful for women who cannot tolerate bisphosphonates or in whom estrogen replacement is considered for other medical reasons. However, the effect of estrogen on the incidence of hip fractures and responsiveness in other tissues in relation to the T262C polymorphism needs to be further investigated.

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		T262C polymorphism		
		T/T	T/C	C/C
PvuII polymorphism	**pp**	48	7	
	Pp	12	38	3
	PP	1	4	16

Table 1 Cross tabulation of subjects according to the PvuII and the T262C polymorphisms. The T262C polymorphism was in linkage disequilibrium with the PvuII intronic polymorphism ($P < 0.001$).

	T262C polymorphism			
	T/T (n = 41)	T/C (n = 36)	C/C (n = 19)	P-value
Age (years)	58.6 ± 1.1	60.2 ± 1.5	59.6 ± 2.2	NS
Body weight (kg)	58.8 ± 1.6	54.9 ± 2.3	56.1 ± 1.9	NS
Duration of menopause (years)	9.1 ± 1.1	11.4 ± 1.4	13.1 ± 2.6	NS
L_{2-4} BMD (g/cm^2)	0.94 ± 0.03	0.90 ± 0.03	0.86 ± 0.04	NS
Femoral neck BMD (g/cm^2)	0.82 ± 0.02	0.76 ± 0.02	0.72 ± 0.03	< 0.05

Table 2 Baseline characteristics of subjects according to the T262C polymorphism. Using ANOVA, there was a difference in femoral neck BMD while no difference in other variables was detected.

	Percent change in BMD			
	L2-4	**p-value**	**Femoral neck**	**P-value**
0.3 mg CEE (n = 51)	5.7 ± 1.0	< 0.0001	1.7 ± 1.0	NS
0.625 mg CEE (n = 45)	6.7 ± 1.1	< 0.0001	1.8 ± 0.9	NS

Table 3 Change in vertebral and femoral BMD after 2-year treatment with 0.3 mg or 0.625 mg CEE. Compared to baseline values. 0.3 mg and 0.625 mg CEE caused a significant increase in vertebral BMD while no change in femoral neck BMD was found.

	Percent change in BMD			
	L2-4	**P-value**	**Femoral neck**	**P-value**
T/T (n = 41)	5.8 ± 1.1	< 0.0001	-0.4 ± 1.0	NS
T/C (n = 36)	6.3 ± 1.3	< 0.0001	1.9 ± 1.1	0.1
C/C (n = 19)	6.7 ± 1.9	< 0.01	5.9 ± 1.4	< 0.0001

Table 4 Change in vertebral and femoral BMD according to the T262C polymorphism after 2-year treatment with estrogen . Vertebral BMD increased regardless of genotype. Femoral neck BMD increased only in those with the T262C polymorphism.

		Sum of squares	df	Mean square	F	P-value
Covariate	**Baseline L2-4 BMD**	326.67	1	326.67	6.08	< 0.05
Main Effects	**T262C genotype**	25.73	2	12.86	0.42	NS
	PvuII genotype	62.54	2	31.27	0.58	NS
	Doses of CEE	34.70	1	34.70	0.65	NS
Model		429.73	6	71.62	1.33	NS
Residual		4779.39	89	53.70		
Total		5209.12	95	54.83		

Table 5 Effects of ERα polymorphisms, doses of CEE and baseline vertebral BMD on the change in BMD after treatment with CEE. Baseline vertebral BMD affected the response to estrogen while no effect of the ERα polymorphisms or doses of CEE was found.

		Sum of squares	df	Mean square	F	P-value
Covariate	**Baseline femoral neck BMD**	356.90	1	356.90	10.17	< 0.01
Main Effects	**T262C genotype**	422.30	2	211.15	6.02	< 0.01
	PvuII genotype	145.09	2	72.54	2.07	NS
	Doses of CEE	0.001	1	0.001	0	NS
Model		1006.69	6	167.78	4.78	< 0.0001
Residual		3123.70	89	35.10		
Total		4130.39	95	43.48		

Table 6 Effects of ERα polymorphisms, doses of CEE and baseline femoral neck BMD on the change in BMD after treatment with CEE. The T262C polymorphism and baseline femoral neck BMD independently affected the response to estrogen. No effect of the PvuII polymorphism or doses of CEE was found.

ASSOCIATION OF A G2014A TRANSITION IN EXON 8 OF ESTROGEN RECEPTOR-α GENE WITH POSTMENOPAUSAL OSTEOPOROSIS

Osteoporosis is under genetic determination as shown in studies performed in daughter-mother pairs (1) and twins (2). The genetics of osteoporosis is likely to be polygenic with multiple genes each contributing a minor effect (3). Association studies looking at various candidate genes for osteoporosis have yielded conflicting results in various populations (4-7). With regard to estrogen receptor-α (ERα) gene, results from association studies using intronic single nucleotide polymorphisms (SNP) have also been conflicting among various population. In order to further investigate the role of ERα gene in osteoporosis, we have recently attempted to identify other SNPs in the promotor region and exons of ERα gene and have identified a novel G2014A SNP in exon 8 of ERα gene which change the corresponding codon from ACG to ACA. Although the identified SNP is synonymous, it is located 6 nucleotides upstream from the end of the stop codon and is thus likely that the G2014A SNP may possess functional significance by being in linkage disequilibrium with certain regulatory elements in the 3'-untranslated region (3'-UTR) of ERα gene. It is therefore the purpose of the present study to investigate the association of this novel G2014A SNP in ERα with osteoporosis in Thai postmenopausal women.

Materials and Methods

Subjects

Postmenopausal women who were at least 55 years old were recruited by advertising to have bone mineral density screening at Ramathibodi Hospital, Bangkok, Thailand. Subjects who had major illness, secondary causes of osteoporosis including glucocorticoid excess, hyperthyroidism and hyperparathyroidism were excluded. All were ambulatory and did not take estrogen or other antiresorptive agents. Before the study, signed informed consent was obtained.

BMD measurement

Bone mineral densities (BMD) were measured by dual-energy X-ray absorptiometry (Lunar Expert XL, Lunar Corp., U.S.A.). Daily calibration and quality control were done regularly according to the manufacturer's recommendation. BMD at anteroposterior L2-4 and femoral neck were measured in each subject. In vivo coefficients of variation for these sites were 1.2% and 1.6%, respectively. Subjects were classified as osteoporotic if their vertebral or femoral BMD were 2.5 standard deviation lower than the mean value of Thai young females.

G2014A genotyping

Genomic DNA of patients was extracted from peripheral leukocytes. DNA segment containing the G2014A SNP site was amplified by PCR with the following primers: forward, 5'-GACGGACCAAAGCCACTTGG and reverse, 5'-CGTGTGGGAGCCAGGGAGCT. The final reaction contained 0.5 μg DNA, 1 unit of Taq polymerase, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$. The reaction was run for 30 cycles with denaturation at 95 °C for 30 seconds, annealing at 67 °C for 60 seconds and extension at 72 °C for 60 seconds. The 124 bp DNA fragments was then subjected to digestion with BtgI restriction endonuclease. In the presence of the G allele, the digestion yielded a 94 and a 31 bp fragments.

Measurement of residual estradiol concentrations

Circulating residual estradiol (E_2) levels were assayed by a ultrasensitive radioimmunoassay with the lower limit of detection of 5 pmol/L (CIS Bio International, France).

Statistical analysis

Difference in genotype distribution was assessed by chi-square test. Stepwise multiple logistic regression was used to identify factors associated with the presence of osteoporosis. Relations between BMD and various factors were determined by multiple linear regression analysis. Data were expressed as mean ± SEM.

Results

The clinical characteristic of the subjects were shown in Table 1. Subjects with osteoporosis had longer duration since menopause and lower body weight compared to those without osteoporosis. No difference in age or circulating E_2 was detected. Most of the women (96.2%) had vertebral osteoporosis while 26.7% had femoral osteoporosis and 22.9% had osteoporosis at both skeletal sites.

Table 2 shows the genotype distribution of the G2014A SNP. Among subjects with osteoporosis 7.5% were homozygous for the A2014 allele and 37.7% were heterozygous. The A2014 allele frequency in this group of subjects was 26.4 %. In subjects without osteoporosis 1.6% were homozygous for the A2014 allele, 27.0% were heterozygous and the A2014 allele frequency in this group of subjects was 15.2%. The genotype distribution in subjects with and without osteoporosis was significantly different with $P < 0.05$.

As shown in Table 3, it was found by stepwise logistic regression analysis that factors associated with the presence of osteoporosis were lower body weight and longer duration since menopause. The G2014A genotype was independently associated with osteoporosis with OR = 2.7 per each A2014 allele. Serum residual E_2 were not associated with the presence of osteoporosis.

As shown in Table 4, in a multiple linear regression model with age, the number of years since menopause, body weight, serum E_2 and the G2014A genotype as independent variables, it was found that factors associated with L2-4 BMD in subjects with osteoporosis were body weight and serum E_2. Besides these two factors, the G2014A genotype was also independently correlated to vertebral BMD in subjects with osteoporosis with the standardized regression coefficient of –0.29 ($P < 0.001$). However, in subjects without osteoporosis, factors associated with vertebral osteoporosis were only body weight and serum E_2. Likewise, at the femoral neck, the relation of the G2014A genotype and BMD was found only in women with osteoporosis ($P < 0.05$) as demonstrated in Table 5.

Discussion

Studies regarding ERα gene as a susceptibility gene for osteoporosis using previously reported intronic SNPs have yielded conflicting results (4-7). The reason for the discrepancy is unclear but is likely to be related to the differences in study design as well as ethnic and environmental factors. Moreover, it is likely that these intronic SNPs are not directly responsible for the association seen in various studies since the functional significance of these intronic SNPs is unclear. In this study, we have demonstrated an association between a novel exonic G2014A SNP in ERα gene and postmenopausal osteoporosis in Thais. The G2014A SNP was also associated with BMD only in subjects with osteoporosis suggesting an interaction between the G2014A SNP and other contributing factors in the determination of bone mass. There have been studies showing such genetic interaction (6, 8, 9) and it is

conceivable that other genetic or environmental factors may possess a permissive role for the effect of the G2014A SNP to be fully exerted. The nature of these interacting determinanats is unknown and warrants further investigations.

Since the G2014A SNP is synonymous, the underlying mechanism of the observed relation is not readily apparent. However, one of the possibilities is that the G2014A SNP is in linkage disequilibrium with regulatory sequences in the 3'-UTR of ERα gene. Both 5'- and 3'-UTR have been found to be important in the regulation of gene expression. ERα gene, similar to other genes coding for steroid hormone receptors, has exceptionally long 3'-UTR. In the 3'-UTR region, AU(AT)-rich element plays modulatory role in the stability of mRNA (10) and polymorphism in the 3'-UTR in the vicinity of the ATTTA motifs has been demonstrated to affect gene transcription as well as certain clinical features (11). Moreover, the 3'-UTR also contains sequences regulating polyadenylation which affect mRNA processing. Variations in the nucleic acid sequence surrounding these polyA signals may be another mechanism through which the G2014A SNP exerts its effect.

Genetic susceptibility to osteoporosis may express during the accrual of peak bone mass or later at the time when postmenopausal bone loss occurs. A number of studies have demonstrated the heritability of bone mass before the attainment of peak bone mass (12, 13) and this is likely to occur before puberty. Susceptibility genes shown to be associated with bone mass during childhood and adolescent include ERα gene (14), VDR gene (15) and type 1 collagen gene (16), although not being without dispute (17). In contrast, studies demonstrated heritability of age-related bone loss and the genes involved are relatively few. In this regard, VDR gene has been shown to be associated with postmenopausal bone loss (18, 19). The role of collagen type 1 gene on postmenopausal is controversial (20, 21). Although the present study has demonstrated the association of the G2014A SNP with postmenopausal osteoporosis, it is still unknown whether the finding is the result of the difference in bone mass since childhood or the effect on postmenopausal bone loss. Further studies in this regard are required to elucidate the mechanism and confirm the nature of the association.

In conclusion, we have demonstrated in this study that a G2014A transition close to the stop codon in exon 8 of ERα gene is associated with osteoporosis in Thai postmenopausal women. Since the SNP is synonymous, it is likely that there is a linkage disequilibrium between the SNP and certain regulatory sequences in the 3'-untranslated region of ERα gene. The finding, however, needs to be reassessed and confirmed in other population with different ethnicity or environmental background.

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	Osteoporotic (n = 106)	Non-osteoporotic (n = 122)	P
Age (year)	65.3 ± 0.5	64.3 ± 0.4	NS
Years since menopause (year)	17.7 ± 0.6	13.1 ± 0.5	< 0.0001
Body weight (kg)	54.8 ± 0.8	60.3 ± 0.8	< 0.0001
E_2 (pmol/L)	23.8 ± 1.4	23.7 ± 1.4	NS

Table 1 Clinical characteristics of the subjects in the study.

	Osteoporotic (n = 106)	Non-osteoporotic (n = 122)
G/G	58 (54.7%)	87 (71.3%)
G/A	40 (37.7%)	33 (27.0%)
A/A	8 (7.5%)	2 (1.6%)

Table 2 Genotype distributions based on the G2014A SNP in osteoporotic and non-osteoporotic subjects. The genotype distributions were significantly different with the A allele over-represented in subjects with osteoporosis ($P < 0.05$).

Variable	Odds ratio	95% CI
Body weight	0.93	0.89-0.96
Years since menopause	1.12	1.08-1.19
E_2	-	-
G2014A genotype	2.7	1.49-4.76

Table 3 Factors associated with osteoporosis from multiple stepwise logistic regression analysis. The G2014A genotype was related to the presence of osteoporosis independently of body weight and the number of years since menopause. The G/G genotype was coded as 1, G/A and A/A as 2 and 3, respectively.

	Osteoporotic		Non-osteoporotic	
Variable	**Standardized coefficient**	**P**	**Standardized coefficient**	**P**
Body weight	0.22	< 0.05	0.19	< 0.05
Age	-	-	-	-
Years since menopause	-	-	-	-
E_2	0.23	< 0.05	0.23	< 0.05
G2014A genotype	-0.29	< 0.001	-	-

Table 4 Factors associated with L2-4 BMD in subjects with and without osteoporosis. The G2014A genotype was associated with L2-4 BMD only in subjects with osteoporosis. The G/G genotype was coded as 1, G/A and A/A as 2 and 3, respectively.

	Osteoporotic		Non-osteoporotic	
Variable	**Standardized coefficient**	**P**	**Standardized coefficient**	**P**
Body weight	0.23	< 0.05	-	-
Age	-	-	-	-
Years since menopause	-0.32	< 0.001	-0.28	< 0.01
E_2	-	-	-	-
G2014A genotype	-0.18	< 0.05	-	-

Table 5 Factors associated with femoral neck BMD in subjects with and without osteoporosis. The G2014A genotype was associated with femoral BMD only in subjects with osteoporosis. The G/G genotype was coded as 1, G/A and A/A as 2 and 3, respectively.

EFFECT OF ESTROGEN REPLACEMENT ON GLUCOSE SENSITIVITY, SERUM LIPIDS AND BONE MARKERS IN HYPOGONADAL MALES

Estrogen possesses several physiologic roles in females. In men, however, the role of estrogen in normal physiology is not entirely clear. Although generally considered a female hormone, estrogen has recently been found to affect bone metabolism in men. For examples, estrogen resistance due to a nonsense mutation in estrogen-receptor-α gene and estrogen deficiency due to mutations in the gene encoding aromatase enzyme have been associated with osteoporosis (1,2). Moreover, the glucose intolerance and dyslipidemia as clinical features in some of these patients. These findings raise the possibility that estrogen may have modulating role in bone, glucose and lipid metabolism in men as well as in women.

Testosterone replacement has been the mainstay treatment of male hypogonadism. Although effective, some hypogonadal males on testosterone replacement still do not have features associated with hypogonadism reversed after testosterone replacement. Long-term replacement of testosterone leads to normalization of bone mineral density (BMD) in a prospective study (3). It is unclear, however, that this effect is due to the replenishment of testosterone per se or the increase in circulating estrogen from peripheral aromatization of testosterone. It is therefore the purpose of the present study to prospectively evaluate the effect of low-dose estrogen on bone, glucose and lipid metabolisms in hypogonadal males.

Materials and Methods

Subjects

Subjects included 13 hypogonadal males on testosterone replacement therapy at Ramathibodi Hospital, Bangkok, Thailand. The study was approved by the local Ethical Committee and all subjects gave informed signed consent prior to the study. Before the study all subjects were being treated with intramuscular testosterone enanthate every 3 to 4 weeks. Subjects were informed to withhold testosterone injection for at least 8 weeks before participating in the study. Then the subjects took 0.3 mg oral conjugated equine estrogen (CEE) daily for 4 weeks. Compliance to the medication was assessed by tablet counting at week 4 and by the percentage increase in serum estradiol concentrations at the end of the study. Four subjects were probably non-compliant at end of the study according to the above criteria and were excluded from the analysis.

Assessments

Assessments of insulin sensitivtity and glucose effectiveness indices were performed at baseline and after 4 weeks of CEE by using the reduced protocol of frequently sampled intravenous glucose tolerance test (FSIVGTT) as previously described (4). Serum samples were also obtained at the beginning and the end of the study for the assessment of serum lipids by colorimetry. Serum LDL-cholesterol (LDL-C) was calculated by the Friedwald's formula. Bone turnover was assessed by serum C-terminal extension peptide of type 1 collagen (CTX) levels by enzyme immonoassay (Roche Diagnostics, Germany) before and after the 4-week period.

Statistical analysis

Changes in parameters compared to baseline values were analyzed by paired-t test. Data were expressed as mean ± SEM.

Results

Table 1 demonstrates the clinical characteristics of the subjects. The age ranged between 22 years to 70 years with a mean age of 37.3 ± 5.0 years. After 8 week since last testosterone injection and before starting CEE, the levels of circulating estradiol were 25.0 ± 5.0 pmol/L. After the 4-week period of CEE administration, serum E2 levels rose significantly to 66.1 ± 14.0 pmol/L ($P < 0.01$).

At baseline, there was a correlation between serum E_2 concentrations and serum insulin (and plasma glucose levels (Table 2). The correlation of E_2 to SI almost reach statistical significance ($P = 0.07$). No correlation of E_2 to other variables was found.

Changes in various parameters after CEE were shown in Table 3. SI did not change significantly after the administration of CEE ($P = 0.09$). Likewise, no change in AIRg was detected. However, SG significantly decreased after CEE ($P < 0.05$). No significant change in serum TC, LDL-C, HDL-C or TG was detected. In regard to bone turnover, serum CTX significantly decreases after CEE administration ($P < 0.05$).

Discussion

The role of estrogen in insulin sensitivity is inconclusive. Patients with Turner's syndrome had deteriorated glucose tolerance after treatment with estrogen combined with norethisterone (5). In women with premature ovarian failure, no change in insulin sensitivity was observed during treatment with estrogen alone. However, there was an improvement in insulin sensitivity after medroxyprogesterone acetate was added (6). Results from the PEPI study have shown that 0.625 mg of CEE modestly decreased fasting levels of insulin and glucose. However, an increase in post-challenge glucose concentrations was observed (7). Regarding SG, it has been increasing recognized that non-insulin mediated glucose uptake play important role in carbohydrate metabolism. Besides abnormality in insulin sensitivity, subjects with impaired glucose tolerance and elderly diabetic patients also had an impairment in SG (8, 9) and both SI and SG has comparable contributing role to glucose disposal rate (10). In a study of a small number of postmenopausal women, oral estradiol but not transdermal was found to decrease insulin levels without effects on SI or SG (11). In the present study, the effect of CEE in increasing SI approached statistical significance. By decreasing SG, the effect of CEE on this parameter appeared to be in the opposite direction to that of SI. The opposing effect on SG may partly explain the inconsistency regarding the effect of estrogen on the levels of glucose in various studies.

Beneficial effects of estrogen in postmenopausal women include favorable profile of serum lipid levels (4). Moreover, estrogen also possesses vasodilatation effect which may contribute to the risk reduction unrelated to serum lipid concentrations (12). In men, however, data regarding serum lipid concentration after estrogen replacement are few. In elderly men with prostatic cancer, high-dose parenteral estrogen caused a decrease and an increase in LDL-C and HDL-C, respectively (13). In term of the relation between sex steroids and coronary heart disease in men, it has been found in cross sectional studies that low serum testosterone levels are associated with increased coronary risk (14, 15). It is unclear, however, that the adverse effect of low circulating testosterone is due to the direct effect of testosterone per se or the its indirect effect by being aromatized to estradiol in peripheral tissues. Nevertheless, high circulating estrogen levels in males has been found to be related to increased rather than decreased coronary risk. In the present study we could not demonstrate the lipid-lowering effect of estrogen in hypogonadal males. It is likely that circulating levels of estrogens has minor effect on

serum lipids in men as opposed to the favorable effect of pharmacologic doses. Despite the lack of effect on serum lipid concentrations, 0.625 mg of CEE decreased bone resorption marker in the present study. A number of cross-sectional studies have demonstrated association of BMD with serum estradiol levels which may be related to the decrease in bone resorption as happen in females. Although high-dose parenteral estrogen retarded bone resorption and preserved bone mass in men with prostatic cancer, the effect of oral conventional doses of estrogen is unknown. The findings suggest that conventional doses of estrogen in men may be beneficial to bone but are likely to have no effect on serum lipid.

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Age (year)	37.3 ± 5.0
Body weight (kg)	66.5 ± 2.4
Fasting glucose (mg/dl)	87.2 ± 9.3
Fasting insulin (μU/ml)	23.2 ± 7.3
SI ($10^4 min^{-1}$ per μU/ml)	1.94 ± 0.58
SG (min^{-1})	0.029 ± 0.003
AIR (μU/ml x min)	577.4 ± 158.1
Total cholesterol (mg/dl)	195 ± 11.3
LDL-C (mg/dl)	129.6 ± 9.2
HDL-C (mg/dl)	33.6 ± 2.0
Triglyceride (mg/dl)	159.2 ± 21.2
CTX (pmol/L)	274.4 ± 59.2

Table 1 Baseline characteristics of the subjects.

Variables	R	P-value
Insulin	0.74	< 0.05
Glucose	0.70	< 0.05
SI	-0.64	0.07
SG	-0.16	NS
AIRg	0.06	NS
TC	-0.22	NS
LDL-C	-0.28	NS
HDL-C	-0.23	NS
TG	0.13	NS
CTX	-0.40	NS

Table 2 Correlation of baseline serum E_2 concentrations with parameters in glucose, lipid and bone metabolism. Serum E_2 correlated to serum insulin and plasma glucose at baseline. No correlation to other varibles was found.

Variables	Before CEE	After CEE	P-value
Insulin (μU/ml)	23.2 ± 7.9	20.2 ± 5.2	NS
Glucose (mg/dl)	80.2 ± 9.3	84.8 ± 6.0	NS
SI ($10^4 min^{-1}$ per μU/ml)	1.94 ± 0.58	2.21 ± 0.58	0.09
SG (min^{-1})	0.029 ± 0.003	0.024 ± 0..002	< 0.05
AIR (μU/ml x min)	577.4 ± 158.1	615.1 ± 105.3	NS
Total cholesterol (mg/dl)	195.0 ± 11.3	194.7 ± 12.3	NS
LDL-C (mg/dl)	129.6 ± 9.2	129.1 ± 12.2	NS
HDL-C (mg/dl)	33.6 ± 2.0	34.0 ± 2.5	NS
Triglyceride (mg/dl)	159.2 ± 21.2	157.9 ± 19.3	NS
CTX (pmol/L)	274.4 ± 59.2	242.0 ± 54.9	< 0.05

Table 3 Changes in parameters after 4-week treatment of CEE. There was a significant decrease in SG ($P < 0.05$). The decrease in SI did not reach statistical significance ($P = 0.09$). Serum CTX decreased ($P < 0.05$) while no change in serum lipid concentrations was found.

CIRCULATING ESTRADIOL AND ESTROGEN RECEPTOR GENE POLYMORPHISMS ARE NOT RELATED TO IDIOPATHIC OSTEOPOROSIS IN ELDERLY MEN

Osteoporosis occurs in men as well as in women, although to a lesser extent. Men have higher peak bone mass and bigger bone than women and this may be accountable for the lower incidence of fractures despite similar rate of bone loss in men and women. Estrogen play important role in bone metabolism in males as well as in females. Smith et al. described a male patient with estrogen insensitivity due to a nonsense mutation in estrogen receptor alpha gene (1). Among others, the clinical features of the patient include osteoporosis. Since then supporting evidences of the skeletal effect of estrogen in males have been accumulating with reports in males with aromatase deficiency (2) and also cross-sectional study in aging males (3). Although estrogen is now believed to have bone modulating effect in men as well as in women, it is unclear whether men with idiopathic osteoporosis has low circulating estrogen as a etiologic factors. Moreover, with regard to the genetics of osteoporosis, it is still unclear whether osteoporosis in men has similar predisposing genetic factors as in women. Since estrogen receptor gene locus has been associated with osteoporosis in a number of studies and we have recently described a G2014A single nucleotide polymorphism in estrogen-receptor-α (ERα) gene which is associated with postmenopausal osteoporosis, It is therefore the purpose of the present study to investigate the role of low circulating estrogen together with the Era gene locus in the manifestation of idiopathic male osteoporosis.

Subjects and Methods

Subjects

Subjects consisted of 98 males aged 60 years or more, of whom 18 had vertebral or femoral osteoporosis based on BMD criteria. Of those with osteoporosis, serum calcium levels and complete blood count were within normal limit. Hypogonadism was excluded based on physical examination and serum total testosterone levels. None has clinical features suggestive of glucocorticoid excess or thyrotoxicosis. Urinary calcium excretion was assessed from 24-hour urine samples and hypercalciuria was also excluded. None was taking medication which may affect calcium or bone metabolism.

BMD measurement

Bone mineral densities (BMD) were measured by dual-energy X-ray absorptiometry (Lunar Expert XL, Lunar Corp., U.S.A.). Daily calibration and quality control were done regularly according to the manufacturer's recommendation. BMD at anteroposterior L2-4 and femoral neck were measured in each subject. In vivo coefficients of variation for these sites were 1.2% and 1.6%, respectively. Subjects were classified as osteoporotic if their vertebral or femoral BMD were 2.5 standard deviation lower than the mean value of Thai young males.

G2014A genotyping

Genomic DNA of patients was extracted from peripheral leukocytes. DNA segment containing the G2014A SNP site was amplified by PCR with the following primers: forward, 5'-GACGGACCAAAGCCACTTGG and reverse, 5'-CGTGTGGGAGCCAGGGAGCT. The final reaction contained 0.5 μg DNA, 1 unit of Taq polymerase, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$. The reaction was run for 30 cycles with denaturation at 95 °C for 30 seconds, annealing at 67 °C

for 60 seconds and extension at 72 °C for 60 seconds. The 124 bp DNA fragments was then subjected to digestion with BtgI restriction endonuclease. In the presence of the G allele, the digestion yielded a 94 and a 31 bp fragments.

T262C genotyping

The T262C nucleotide change in exon 1 was genotyped by allele-specific PCR using a common primer, 5'TTGCTGCTGTCCAGGTACAC and 2 specific primers, 5'CCCTCCACACCAAAGCATCT and 5'CCTCCACACCAAAGCATCC. The final reaction contained 0.5 μg DNA, 1 unit of Taq polymerase, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1 mM $MgCl_2$. The reaction was run for 30 cycles with denaturation at 95 °C for 30 seconds, annealing at 63 °C for 30 seconds and extension at 72 °C for 30 seconds.

Measurement of residual estradiol concentrations

Circulating residual estradiol (E_2) levels were assayed by a ultrasensitive radioimmunoassay with the lower limit of detection of 5 pmol/L (CIS Bio International, France).

Statistical analysis

Differences in genotype distribution and other continuous variables were assessed by chi-square test and Student's t-test, respectively. Data were expressed as mean ± SEM.

Results

There was no significant difference in age, height and body mass index between those with and without osteoporosis. However, subjects with osteoporosis had lower body weight than those without the disease (56.4 ± 1.3 vs. 64.6 ± 1.2 kg, $p < 0.01$). In terms of circulating estradiol, no significant difference was detected (90.6 ± 12.7 vs. 88.7 ± 5.0 pmol/L).

With regard to SNP in ERα gene, the genotype distributions of the T262C SNP in exon 1 and G2014A SNP in exon 8 did not differ in subjects with and without osteoporosis. In a multiple logistic regression model with age, body weight, height, BMI, serum estradiol levels, estrogen receptor alpha SNP as independent variables, it was found that only body weight (OR 0.86, 95% CI 0.78-0.94 was independently associated with osteoporosis.

Discussion

Although overt estrogen deficiency or dysfunction can cause osteoporosis in men as demonstrated in males with estrogen resistance or aromatase deficiency, the role of mild relative estrogen deficiency in elderly men with idiopathic osteoporosis is less clear. Results from cross-sectional studies in populations at large have mostly demonstrated an association between circulating estrogen and bone mass (3, 4). Relatively higher significance of estrogen over that of androgen has also been demonstrated in some studies (5). Based on these findings, we postulated that relative estrogen deficiency may play etiologic role in males with idiopathic osteoporosis. However, on the contrary, no difference in circulating estradiol concentrations was found between men with and without idiopathic osteoporosis. The finding suggests that although estrogen deficiency can induce bone loss in both women and men, the bone loss caused by relative estrogen deficiency in men may

not be high enough to cause osteoporosis in elderly men. In fact, estrogen appears to have relatively little effect on bone turnover in elderly men as compared to young men (6).At least in experimental animals, bone appears to have a sexually dimorphic response to aromatase deficiency (7).Unlike female rats whose bone turnover increases in response to aromatase deficiency, the response in male rats is reversed. It is likely that osteoporosis in males is pathogenetically different from that in females. Other as yet undefined age-related factors are likely to have greater bone-mass-lowering effect superimposed on that of estrogen deficiency and cause osteoporosis in predisposed subjects.

In keeping with the lack of the role of estrogen in male idiopathic osteoporosis, ERα gene locus as assessed by SNPs in exons 1 and 8 also yielded no relation with male idiopathic osteoporosis despite associations found in our previous cross-sectional study (5) and a study in boys (8). At the cellular level, decreased expression of ERα has been demonstrated in the osteoblasts of men with idiopathic osteoporosis (9).The sample size in the present study was small and the minor role of ERα gene locus, if any, cannot be totally excluded. The only factor associated with osteoporosis in the present study was low body weight. This is in accordance with previous cross-sectional studies showing a relation between body weight and BMD in men (10, 11). The increased risk of osteoporosis in men with low body weight may be due to the increase in the rate of bone loss. In a recent study, elderly men was found to lose bone by 0.2-3.6% per year and body weight had a positive correlation with the rate of bone loss (12). The mechanism through which low body weight brings about greater bone loss is at present unclear.

In conclusion, although estrogen affect bone metabolism in males, lower circulating estradiol together with the estrogen receptor gene locus are not major causes of osteoporosis in males.

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Output จากโครงการ

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ

1. Ongphiphadhanakul B, Chanprasertyothin S, Payatikul P, Sae Tung S, Piaseu N, Chailurkit L, Chansirikarn S, Puavilai G, Rajatanavin R. Oestrogen-receptor-alpha gene polymorphism affects response in bone mineral density to oestrogen in post-menopausal women. Clin Endocrinol (Oxford) 2000;52:581-585.
2. Ongphiphadhaankul B, Chanprasertyothin S, Payattikul P, Saetung S, Piaseu N, Chailurkit L, Chansirikarn S, Puavilai G, Rajatanavin R. Association of a T262C transition in exon 1 of estrogen-receptor-α gene with skeletal responsiveness to estrogen in postmenopausal women. Submitted to Journal of Endocrine Investigation
3. Ongphiphadhaankul B, Chanprasertyothin S, Payattikul P, Saetung S, Piaseu N, Chailurkit L, Rajatanavin R. Association of a G2014A transition in exon 8 of estrogen-receptor-α gene with postmenopausal osteoporosis. Submitted to Osteoporosis International

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Oestrogen-receptor-α gene polymorphism affects response in bone mineral density to oestrogen in post-menopausal women

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Summary

OBJECTIVE An oestrogen-receptor-α (ERα) gene polymorphism has been variably reported to be related to bone mass. To investigate whether this ERα gene polymorphism is associated with a functional difference, we assessed the response in bone mineral density (BMD) to oestrogen therapy in post-menopausal women in relation to ERα gene polymorphism.

PATIENTS AND MEASUREMENTS Subjects consisted of 124 Thai post-menopausal women. Sixty-three of the women were less than 6 years post-menopausal and 61 were more than 10 years post-menopausal with vertebral or femoral osteoporosis as defined by BMD T-score less than −2·5. Subjects were randomly allocated to receive 0·3 mg ($n=67$) or 0·625 mg ($n=57$) of conjugated equine oestrogen (CEE). All subjects also took 5 mg medroxyprogesterone acetate. Vertebral and femoral neck BMD were measured at baseline and 1 year after treatment. Data were expressed as mean ± SEM. Capital P represents the absence of the restriction site while lower-case p indicates the presence of the restriction site.

RESULTS For subjects on 0·625 mg CEE, BMD at L2–4 increased significantly after 1 year in those with pp ($n=20$) Pp ($n=29$) and PP genotypes ($n=8$) ($P<0·001$). However, in subjects on 0·3 mg CEE, BMD at L2–4 increased significantly after 1 year in subjects with Pp ($n=34$, $+7·6 \pm 1·5\%$, $P<0·001$) and PP genotypes ($n=13$, $+6·9 \pm 1·0\%$, $P<0·001$), but not in those with pp genotype ($n=20$, $+2·3 \pm 2·1\%$, $P=$ NS). After adjusting for age and years since menopause, the change in vertebral BMD was still lower in those without the P allele compared to those with the P allele ($P<0·05$). Femoral BMD did not significantly change regardless of dose of CEE and genotype.

CONCLUSIONS We conclude that ERα gene polymorphism affects skeletal response to oestrogen in post-menopausal women. The effect of ERα gene polymorphism appears to be site-specific and does not relate to biochemical markers of bone turnover. Determination of ERα genotype may help identify post-menopausal women who will have more skeletal benefit from oestrogen therapy.

Osteoporosis is partly genetically determined. A number of candidate genes for osteoporosis have been examined. For example, it was found that subjects with the bb vitamin D receptor (VDR) genotype have higher bone mineral density (BMD) compared with other genotypes (Morrison *et al.*, 1994). However, a number of subsequent studies were unable to confirm such an association (Peacock, 1995). Nevertheless, the VDR polymorphism has been shown to be associated with functional differences in terms of intestinal calcium absorption and suppression of parathyroid hormone levels after treatment with calcitriol (Dawson-Hughes *et al.*, 1995; Howard *et al.*, 1995; Gennari *et al.*, 1997). Besides VDR polymorphisms, oestrogen-receptor-α (ERα) gene polymorphisms have also been associated with BMD in some (Kobayashi *et al.*, 1996; Mizunuma *et al.*, 1997; Ongphiphadhanakul *et al.*, 1998) but not all studies (Han *et al.*, 1997; Gennari *et al.*, 1998). The ERα gene polymorphisms studied thus far reside in introns and their functional significance is unclear. A previous study could not demonstrate the association between skeletal responsiveness to oestrogen and ERα genotype (Han *et al.*, 1997). However, the doses of oestrogen used was not clearly stated. If ERα gene polymorphisms do affect skeletal response, it is likely that such differences may be more readily elicited at lower doses of oestrogen. It is therefore the purpose of the present study to assess prospectively the difference in skeletal response to oestrogen replacement in relation to ERα genotypes and doses of oestrogen in post-menopausal women.

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Materials and methods

Subjects

Subjects consisted of 124 post-menopausal women; 63 were less than 6 years post-menopausal (group 1) while 61 were more than 10 years post-menopausal with osteoporosis as defined by lumbar or femoral neck BMD lower than −2·5 standard deviations from the mean of Thai young women (group 2). All subjects did not smoke or drink and did not engage in regular strenuous exercise. The subjects were recruited by flyers or direct contact. All subjects gave informed consent and the study was approved by the ethical clearance committee on human rights related to research involving human subjects of the Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Thailand.

Medications

Subjects were allocated randomly to one of the two treatments, 0·3 or 0·625 mg of conjugated equine oestrogen (CEE). Subjects in group 1 with an intact uterus were also given 5 mg of medroxyprogesterone acetate from days 1–12 each month while those in group 2 took the drug daily. Each subject also took 750 mg elemental calcium supplementation in the form of calcium capsules daily. The compliance to medication was confirmed by counting the remaining tablets at each visit.

Bone mineral densitometry

BMD was measured by dual-energy X-ray absorptiometry (Lunar Expert XL, Lunar Corp., USA). Daily calibration and quality control were performed regularly according to the manufacturer's recommendation. BMD at anteroposterior L2–4 and femoral neck were measured in each subject. *In vivo* coefficients of variation for these sites were 1·2% and 1·6%, respectively.

Laboratory assays

Fasting blood samples were obtained from subjects between 0800 and 1000 h. Serum samples were frozen at −20°C until measurement. Serum intact osteocalcin (OC) was determined by enzyme immunoassay (Metra Biosystems, USA). Twenty-four-hour urine was collected in each subject. Urinary calcium and creatinine were determined by standard methods. Urinary deoxypyridinoline crosslink (DPD) were assessed by enzyme immunoassay (Metra Biosystems, USA). The intra-assay coefficients of variation for OC and DPD were 12·9% and 5·2%, respectively.

ERα genotyping

Genomic DNA was extracted from peripheral leucocytes by phenol/chloroform extraction. DNA sequence flanking the polymorphic site in intron 1 of ERα gene was amplified by polymerase chain reaction with the following primers: forward, 5′CTGCCACCCTATCTGTATCTTTTCCTATTCT CC; and reverse, 5′TCTTTCTCTGCCACCCTGGCGTCGAT TATCTGA (Kobayashi *et al.*, 1996). The 50 μl final reaction contained 0·1 μg DNA, 1 unit of Taq polymerase, 10 mM Tris-HCl, pH 8·3, 50 mM KCl, 1 mM $MgCl_2$, 200 μM each of the four deoxyribonucleotides and 0·4 μM each of the primers. The reaction was run for 30 cycles with denaturation at 94°C for 30 s, annealing at 61°C for 40 s and extension at 72°C for 90 s. The final PCR product was then digested with *Pvu II* restriction endonuclease. The digested material was resolved on 1·4% agarose gel with ethidium bromide staining. Given the repeatability of the assay (100% in 20 samples), the assay was run once, unless ambiguous, without an internal control for each sample. The results were read without the knowledge of BMD and treatment data. Capital P represents the absence of the restriction site while lower-case p indicates the presence of the restriction site.

Statistical analyses

Data were expressed as mean ± SEM. Two-way ANOVA was used to assess the combined effects of the dose of CEE and ERα genotype on the changes in BMD. Differences in independent variables were analysed by one-way ANOVA or ANCOVA. Changes in BMD and biochemical markers of bone turnover were assessed by paired Student's *t*-test.

Results

The genotype distribution was as follows: pp 40 (32·3%), Pp 63 (50·8%) and PP 21 (16·9%) which conformed to the Hardy–Weiberg equilibrium. As shown in Table 1 there was no difference in age, years since menopause, body weight and biochemical markers of bone turnover among subjects with different genotypes. Although BMD at L2–4 and femoral neck tended to be lower in subjects with PP genotype, this did not reach statistical significance. Similarly, no difference in baseline characteristics in relation to the doses of CEE was found.

When assessing the difference in the change in BMD after oestrogen in relation to doses and genotype, it was found by two-way ANOVA that there is a significant effect of the dosage of CEE ($P<0·05$) but the ERα genotype was of no effect ($P=0·17$). However, there was a two-way interaction between genotype and the dosage of oestrogen ($P<0·05$). Therefore the

Table 1 Baseline characteristics (mean ± SEM) of subjects in relation to ERα genotype and the dose of CEE

	Genotype				Dose of CEE		
	pp ($n=40$)	Pp ($n=63$)	PP ($n=21$)	P	0·3 mg ($n=67$)	0·625 mg ($n=57$)	P
Age (year)	59·9 ± 1·2	57·9 ± 1·0	59·7 ± 2·0	NS	58·4 ± 1·0	59·4 ± 1·1	NS
Years since menopause (year)	13·1 ± 1·7	8·6 ± 0·9	12·7 ± 2·4	NS	10·9 ± 1·2	11·1 ± 1·2	NS
Body weight (kg)	57·7 ± 1·4	56·7 ± 1·2	55·1 ± 3·2	NS	56·4 ± 1·5	57·2 ± 1·1	NS
L2–4 BMD (g/cm^2)	0·94 ± 0·03	0·94 ± 0·02	0·87 ± 0·04	NS	0·92 ± 0·02	0·94 ± 0·02	NS
Femoral neck BMD (g/cm^2)	0·80 ± 0·03	0·79 ± 0·02	0·73 ± 0·03	NS	0·78 ± 0·02	0·78 ± 0·02	NS
OC (ng/ml)	9·3 ± 1·0	11·1 ± 0·8	10·4 ± 1·4	NS	9·6 ± 0·6	8·9 ± 0·6	NS
DPD (nmole/mmole creatinine)	4·3 ± 0·2	4·8 ± 0·2	4·2 ± 0·4	NS	4·3 ± 0·2	4·9 ± 0·3	NS

Table 2 Changes in L2–4 and femoral neck BMD (mean ± SEM) after 1-year of 0·3 and 0·625 mg of CEE. The P-values of less than 0·05 indicate significant changes compared to baseline values

	0·3 mg CEE					0·625 mg CEE			
	L2–4 BMD		Femoral neck BMD			L2–4 BMD		Femoral neck BMD	
Genotype (n)	% Change	P	% Change	P	Genotype (n)	% Change	P	% Change	P
pp (20)	2·3 ± 2·1	NS	1·6 ± 1·0	NS	pp (20)	8·7 ± 1·6	<0·001	3·5 ± 2·1	NS
Pp (34)	7·6 ± 1·5	<0·001	2·0 ± 1·2	NS	Pp (29)	7·8 ± 1·4	<0·001	2·1 ± 2·0	NS
PP (13)	6·9 ± 1·0	<0·001	2·0 ± 1·2	NS	PP (8)	7·7 ± 2·6	<0·001	−0·1 ± 2·1	NS

Table 3 Change in L2–4, femoral neck BMD and biochemical markers of bone turnover (mean ± SEM) in relation to the doses of oestrogen and ERα genotypes. Values shown were adjusted for effect of age and years since menopause

	0·3 mg CEE			0·625 mg CEE		
	pp genotype ($n=20$)	Pp or PP genotypes ($n=47$)	P	pp genotype ($n=20$)	Pp or PP genotypes ($n=37$)	P
Change in L2–4 BMD (%)	2·8 ± 1·8	7·2 ± 1·2	<0·05	7·8 ± 1·8	8·2 ± 1·3	NS
Chane in femoral neck BMD (%)	1·6 ± 1·3	2·0 ± 0·9	NS	1·6 ± 1·6	2·8 ± 1·2	NS
Change in OC (%)	−11·0 ± 5·5	−23·2 ± 3·5	NS	−6·5 ± 9·7	−18·6 ± 6·8	NS
Change in DPD (%)	−14·3 ± 8·0	−13·6 ± 5·6	NS	−26·9 ± 7·7	−22·6 ± 6·0	NS

analyses were performed separately for each dose of oestrogen. As shown in Table 2, 0·3 mg CEE did not significantly affect vertebral BMD in women with the pp genotype. In contrast, subjects with Pp or PP genotypes had increased vertebral BMD after 1-year treatment with 0·3 mg CEE. There was a significant correlation between ERα genotypes and the proportion of subjects who had 3% or more increase in vertebral BMD after treatment (Spearman's rho = 0·26, $P<0·05$). The proportion increased from 55% (11/20) in pp genotypes to 64·7% (22/34) and 92·3% (12/13) in Pp and PP genotypes, respectively. No change in BMD was demonstrated at the femoral neck regardless of genotype. In contrast to the findings with 0·3 mg CEE, 0·625 mg CEE caused an approximately 8% increase in L2–4 BMD regardless of genotype. As with 0·3 mg CEE, no significant change was demonstrated at the femoral neck.

After correcting for age and the number of years since menopause using analysis of covariance, subjects on 0·3 mg CEE with the P allele still had significantly higher increase in

L2–4 BMD compared to those without the P allele ($P<0.05$). No difference in the change at femoral neck was found. Neither the changes in verterbral nor femoral BMD were different among subjects on 0·625 mg CEE with different genotypes (Table 3). In terms of biochemical markers of bone turnover after adjusting for age and years since menopause, there was no difference in the decrease of serum OC or urinary DPD in relation to the dose of oestrogen or genotype.

Discussion

Pvu II and Xba I RFLPs in intron 1 of ERα gene have been found in some, but not all, association studies to be related to bone mass. Moreover, the direction of change in bone mass in relation to the *Pvu II* RFLP appeared to be different among studies. These conflicting results make the contribution of ERα gene polymorphisms to osteoporosis unclear. Moreover, the polymorphisms studied thus far are located in an intron and are thus of uncertain functional significance. In the present study, we demonstrated that the intronic *Pvu II* RFLP was related to response in vertebral BMD and the relation was evident only with low-dose CEE. The genotype distribution in the present study was similar to previous studies in Thais (Ongphiphadhanakul *et al.*, 1998) and also to that in Caucasians (Gennari *et al.*, 1998). The association between the ERα gene polymorphism and skeletal response suggests that the intronic polymorphism possesses functional significance. However, the effect is minor as suggested by the inability of higher dose of CEE to elicit the difference in response. Confounded by other genetic and environmental factors, the contribution of ERα polymorphism to osteoporosis may be difficult to identify by an association study. Intervention with relevant agents which act through the protein of interest may thus be helpful in investigating the importance of candidate genes in the pathogenesis of osteoporosis. Our finding is consistent with a recent study in Caucasian elderly women, which suggested a relationship between changes in BMD after oestrogen replacement and vitamin D receptor and ERα genotypes (Deng *et al.*, 1998). However, it is different from a study in Korean patients where no association between skeletal response and ERα gene polymorphism was found (Han *et al.*, 1997). Subjects were not separately analysed based on the doses of oestrogen in that study. Doses of medication may need to be considered with respect to the effect of genetic polymorphism on the responses to treatment.

Single nucleotide polymorphisms (SNP) occur throughout the human genome (Wang *et al.*, 1998). Although the function of these nucleotide changes is unclear, they can serve as genetic markers for studying the genetic basis of diseases. Recently, it has been demonstrated that SNP in certain genes may affect drug or dietary responses (Huizenga *et al.*, 1998; Mata *et al.*, 1998). With regard to oestrogen replacement therapy, 0·625 mg of CEE is generally considered to be the optimal dose to prevent bone loss in post-menopausal women. Recently, however, residual endogenous oestrogen in post-menopausal women was demonstrated to be related to fracture risk (Cummings *et al.*, 1998) and the reduction in endogenous oestrogen by aromatase inhibitor can increase bone turnover (Khosla *et al.*, 1997). These findings combined suggest that lower doses of oestrogen may be effective in the preservation of bone mass in post-menopausal women. This is in keeping with previous studies that, in the presence of calcium supplementation, lower doses of oestrogen are protective for bone (Ettinger *et al.*, 1987) without stimulating the endometrium (Genant *et al.*, 1997). The finding in the present study that the response to low-dose oestrogen is related to ERα genotype raises the possibility that lower doses of oestrogen may not be equally effective in all individuals. One of the limitations of our study is the relatively small number of subjects. Power calculations for the change in vertebral BMD in subjects with the pp genotype on low-dose CEE revealed that our sample was adequate to detect a 5% change with 80% power at 95% confidence. However, when compared with the vertebral response in subjects on the higher dose, the extent of change in subjects on 0·3 mg CEE was significantly lower. If the present finding is confirmed in other studies, clinical trials looking at the skeletal effect of low-dose oestrogen should also take ERα genotype into consideration since ERα genotyping may help identify women who would be most likely to respond to low-dose oestrogen. Whether lower doses of oestrogen in those with a favourable skeletal response will possess less side-effects in terms of breast and endometrium stimulation is unknown. Moreover, whether detecting ERα genotype will help in identifying post-menopausal women whose serum lipid levels would respond favourably to low-dose oestrogen remains to be determined.

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