# Association between estrogen receptor a polymorphisms and equol production, and its relation to bone mass

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Abstract. To investigate the relationship between estrogen receptor polymorphisms and equol production and its effect on bone turnover, 139 workers (mean age 38.3±11.1 years) in Japan were recruited. Bone mineral density (BMD), bone turnover markers, and serum equol were measured at a health examination. DNA samples were prepared to detect the estrogen receptor  $\alpha$  (ER $\alpha$ ) polymorphism and were digested by PvuII. The number of equal producers was 57. No statistically significant differences were observed in bone mineral density and bone turnover markers between each ER $\alpha$  polymorphism and equol production. Since the adjusted odds ratio indicated that interaction itself decreased the risk of osteosonoassessment index (OSI) reduction using logistic regression analysis, further analysis was performed divided by each ER $\alpha$ polymorphism. Although the crude odds ratio showed no relationship between equol producers and non-producers, the adjusted odds ratio showed that equal producers with ER $\alpha$  pp had a significantly decreased risk of OSI reduction. Although this study was cross-sectional, both equol production and  $ER\alpha$  polymorphism are closely associated with each other in relation to BMD.

## Introduction

Bone mineral density (BMD), one of the most significant predictors of osteoporotic fracture, is influenced by both environmental and genetic factors (1). Although osteoporosis

results in inadequate skeletal strength predisposing its sufferers to fracture, most osteoporotic patients are diagnosed after the occurrence of fractures because osteoporosis itself lacks specific symptoms. Even if osteoporosis is diagnosed before a fracture occurs, it is difficult to recover BMD in a short period of time. In females, BMD reaches its maximum in adolescence and decreases gradually until menopause, after which it decreases dramatically (2). Since reduction in BMD due to menopause is unavoidable, it seems important to achieve the maximum possible level of BMD in adolescence and to prevent BMD reduction before menopause.

Estrogen deficiency plays a major role in the pathogenesis of postmenopausal osteoporosis. Estrogen deficiency is characterized by increased bone resorption and a remodeling imbalance (3). The restriction fragment length polymorphisms on intron I of the estrogen receptor  $\alpha$  (ER $\alpha$ ) gene are also supposed to affect BMD since the Px haplotype, in which P lacks a PvuII site and x possesses an XbaI site, shows a significant association with low BMD (4). The BMD of the femur and lumbar spine of healthy young men displayed the same tendency (5). On the other hand, recent meta-analysis data suggest that XX of ERa polymorphism may be related to high BMD and low fracture risk (6,7). Thus, the effect of  $ER\alpha$  polymorphism on BMD is still controversial. However, it appears that women with the P allele may benefit more from the protective effect of postmenopausal hormone replacement therapy (HRT) than those with the pp homozygote (8). In addition, this *Pvu*II site is located in a potential binding site for B myb transcription factor (9). Thus, this P/p polymorphism was employed in the present study.

Soy is part of the traditional diet of Asians, and epidemiological data indicate that Asian people have a lower rate of osteoporotic fractures, cardiovascular diseases, postmenopausal symptoms, and certain types of cancers than Western populations (10). Soy isoflavones are often referred to as weak estrogens because genistein is a relative potent agonist for ERß (11). Soy isoflavones, such as genistein, daidzein, and glycitein show estrogenic activity so they are also called phytoestrogens. In humans, equol is produced from daidzein in the gastrointestinal tract (12). Equol is structurally similar

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to 17ß-estradiol, which has an affinity for estrogen receptors so that it possesses estrogenic activity. Dietary equol exerts mild estrogenic effects in the pituitary, uterus, mammary glands, liver and fat tissue, but shows limited bone sparing effects in ovariectomized rats (12). Moreover, the relationship between ER receptor polymorphisms and equol production and its effect on bone turnover is still uncertain.

In the present study, we investigated the relationship between ER polymorphisms and equol production and the effects on bone turnover.

#### Subjects and methods

*Subjects*. A total of 139 workers at local hospitals in Japan were recruited with informed consent. The subjects had a mean age of  $38.3\pm11.1$  years and 22 were male. Both premenopausal and perimenopausal women were included in the study.

This study was approved by the Medical Ethics Committee of Kawasaki Medical School and Kawasaki Medical School Hospital (no. 100).

*Lifestyle factors*. The usual dietary intake was assessed using a food frequency questionnaire according to an 11-foodgroup classification (13). This questionnaire was designed and validated previously to measure frequent food intake for seven days. Daily dietary intakes were calculated from this seven-day weighed-food intake questionnaire. We used the soybean intake calculated by this questionnaire.

The subjects had their heights and weights measured wearing light clothing, and their body mass indexes (BMI) were calculated.

*Bone mineral density measurement*. Bone mineral density was measured by an ultrasound method to obviate X-ray exposure. The speed of sound, transmission index, and osteosono-assessment index (OSI) at the calcaneus were measured by using quantitative ultrasound (QUS) AOS-100 (Aloka Co., Ltd., Tokyo). We used OSI as the bone mineral parameter.

Biochemical markers of bone turnover. The second morning urine specimens were collected, and blood samples were obtained at the same time as QUS measurement. Both serum and urine specimens were stored at -20°C until measurement. Serum bone-specific alkaline phosphatase (BAP) and serum y-carboxylated (Gla) osteocalcin were measured as bone formation markers. Gla was measured using the Gla-type osteocalcin EIA kit (Takara, Osaka, Japan). Osteocalcin correlates well with bone formation, but is readily subject to rapid degradation in serum (14). Since not only Gla-osteocalcin and undercarboxylated osteocalcin (uc-OC) but also fragments of various sizes of them coexist in the circulation, different immunoassays have yielded varying results (15). In the present study, a kit utilizing an antibody specific to 17Gla and its adjacent region was used to isolate Gla. Although  $\gamma$ carboxylation preferentially occurred at residues 21 and 24 (16), 17Gla is essential for a Ca-dependent conformational transition (17), indicating that the 17Gla-specific antibody recognizes fully  $\gamma$ -carboxylated osteocalcin to be active. Deoxypyridinoline (DPD) and type I collagen cross-linked N-telopeptides (NTX) in urine were measured as bone



Figure 1. Representative types of polymorphisms in the estrogen receptor  $\alpha$  (ER $\alpha$ ) gene.

resorption markers. Both were corrected by creatinine in urine. The menstrual cycle of each subject was not considered as the effects of the menstrual cycle on bone turnover are thought to be small (18).

*Measurement of equol*. Serum equol was measured by high performance liquid chromatography (HPLC) according to the method of Hidaka *et al* (19). Although many studies suggest that *Bifidobacterium*, *Escherichia coli*, *Bacteroides ovatus*, *Ruminococcus productus* and *Streptococcus intermedius* could be involved in daidzein metabolism (20,21), the main bacteria responsible for daidzein have not been definitely identified (22). In humans, only 30-50% of individuals are capable of producing equol from daidzein (23). An equol producer was defined as one who had a serum concentration of equol >4 ng/ml.

*ERa polymorphism.* Blood samples were obtained at health examination, and genomic DNA was extracted from leukocytes. Intron 1 of the ER gene, where the *Pvu*II digestion site is located, was amplified using polymerase chain reaction (PCR) (24). After amplification using a set of primers (forward: CTGCCACCCTATCTGTATCTTTTCCTATTCTCC and reverse: TCTTTCTCTGCCACCCTGGCGTCGATTATC TGA), the products were digested with *Pvu*II: p but not P was digested. Fig. 1 shows the ERα polymorphism types.

Statistical analysis. One-way analysis of variance (ANOVA) with Fisher's PLSD as a *post hoc* test was performed to compare differences in lifestyle factors, biochemical markers of bone turnover, and OSI for each ER $\alpha$  polymorphism and equol producer. Two-way ANOVA and a logistic regression test were performed to examine the interaction between ER $\alpha$  polymorphism and equol production statuses. When significant differences were detected, contrast analyses were performed using Sheffe's multiple comparison test. Odds ratios as estimated risk with 95% confidence intervals were calculated by logistic regression using JMP 7.0 (SAS Corp.).

#### Results

Lifestyle factors, equol production, and ER polymorphisms of subjects. No statistical difference in mean age was observed between males and females (Table I). The daily intake of soybean and soy-related foods was ~60 g, which is the same as the Japanese average (25). The mean BMI was ~22, indicating that most subjects were not obese. Bone biochemical markers and OSI of all subjects were within normal limits, indicating that the subjects, including the postmenopausal women, were

	Total	Divided by ER $\alpha$ polymorphism			Divided by equol production	
		PP	Рр	pp	Producer	Non-producer
Number of subjects	139	22	68	49	57	82
Mean age Male (n=22) Female (n=117)	38.3±11.1 38.0±13.4 38.4±10.7	38.9±12.0	39.2±11.5	36.9±10.2	39.9±11.2	37.2±11.0
Soybean intake (g/D)	61.0±38.0	65.9±47.1	66.2±37.2	51.2±33.0	65.9±44.8	57.3±31.9
BMI	21.9±3.0	20.9±2.7	22.5±3.1	21.5±2.7	22.5±3.1	21.5±2.8
Gla (ng/ml)	4.87±2.44	5.23±3.00	4.90±2.63	4.67±1.86	5.06±2.27	4.73±2.56
uc-OC (ng/ml)	2.00±1.02	2.29±1.21	1.79±0.89	2.15±1.06	1.82±0.97	2.12±1.05
BAP (U/l)	23.7±8.4	26.4±10.9	23.5±7.9	22.6±7.7	22.9±8.4	24.2±8.4
DPD (nM/mM Cr)	$6.03 \pm 2.10$	6.79±2.85	5.95±1.83	5.81±2.03	6.15±1.94	5.95±2.21
NTx/Cr (nM BCE/mM Cr)	13.9±4.1	12.5±5.9	13.7±3.7	14.7±3.5	13.7±4.5	14.0±3.8
OSI	2.66±0.29	2.65±0.27	2.67±0.31	2.67±0.28	2.67±0.30	2.66±0.28

Table I. Characteristics of subjects.

Gla, carboxylated osteocalcin; uc-OC, undercarboxylated osteocalcin; BAP, bone-specific alkaline phosphatase; NTX, N-terminal cross linked telopeptide; DPD, deoxypiridinoline; OSI, osteosono-assessment index. No statistical difference was observed in each ERa polymorphism or equol production.

thought to be healthy and did not have osteoporosis or bone disease.

When ER $\alpha$  RFLPs were considered, in good accordance with previous reports (4,26), ~35.3% of subjects had the pp homozygote, 15.8% had the PP homozygote, and 48.9% had the Pp heterozygote. The allele frequency matched the Hardy-Weinberg equation. Among these three groups, no statistical differences were observed in age, daily soy intake, BMI, biochemical markers of bone turnover, and OSI.

On the other hand, 57 (41.7%) subjects produced equol. When subjects were divided by equol production, there were no statistical differences in age, BMI, daily soy intake, biochemical markers of bone turnover, and OSI between equol producers and non-producers. Since the baseline characteristics did not differ between these groups as well as among ER $\alpha$  polymorphism groups, the interactional effect on ER $\alpha$  polymorphisms and equol production was assessed.

Univariate comparison of bone biochemical markers of bone turnover and OSI. When interactional effects on bone formation markers and OSI were assessed using two-way ANOVA (Fig. 2), no statistical differences were observed in OSI, Gla and BAP. However, uc-OC showed an interactional effect, i.e., equol non-producers with PP and equol producers with pp showed high uc-OC levels. Bone resorption markers such as DPD and NTX produced no interactional effects (Fig. 3). These results indicated that equol production and ER polymorphism were correlated with each other. The univariate analysis seemed to be insufficient to exclude confounding factors and to estimate which parameter was associated with the bone mineral density so that further multivariate analyses were performed using logistic regression analysis. Table II. Risk determinants in the reduction of OSI.

	Crude odds ratio (95% CI)	Adjusted odds ratio (95% CI)
ERα		
рр	1.000 (reference)	1.000 (reference)
Рр	1.147 (0.451-2.916)	2.160 (0.646-7.587)
PP	0.913 (0.268-3.179)	0.535 (0.099-2.809)
Equol		
Non-producer	1.000 (reference)	1.000 (reference)
Producer	0.787 (0.397-1.556)	0.797 (0.328-1.944)
Interaction	0.625 (0.259-1.494)	0.008 (0.0001-0.576)

Odds ratios indicating that OSI was lower than 2.66 to higher than 2.66 were calculated according to potential risk factors. Interaction was multiplied by ER $\alpha$  and equol, and adjusted interaction itself reduced the risk (<sup>a</sup>p<0.05). Odds ratios and 95% confidence intervals (CI) were evaluated by adjusted model for age, BMI, daily soy intake and bone turnover markers.

*Logistic regression analysis and risk determinants for bone mineral loss.* Since the average OSI of subjects was 2.66, a logistic regression analysis to clarify possible risk factors was performed when the OSI was <2.66.

The crude odds ratio showed that both  $ER\alpha$  polymorphism and equal production had no association with OSI (Table II). In addition, interaction itself had also no association. The odds ratio adjusted by age, BMI, daily soy intake, and bone



Figure 2. Differences in bone formation markers in each ER polymorphism and equol production. No statistical differences were observed in carboxylated osteocalcin (Gla), bone-specific alkaline phosphatase (BAP), and osteosono-assessment index (OSI). Interactional effect was shown in undercarboxylated osteocalcin (uc-OC) (two-way ANOVA, p<0.05).



Equol producer

Figure 3. Differences in bone resorption markers in each ER polymorphism and equol production. No statistical differences were observed in deoxypiridinoline (DPD) and N-terminal cross-linked telopeptide (NTX) (two-way ANOVA).

Table III. Risk determinants in reduction of OSI divided b	by each ER $\alpha$ polymorphisn
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		PP	Рр	pp		
		Crude odds ratio (95% CI)				
Equol	Non-producer	1.000 (reference)	1.000 (reference)	1.000 (reference)		
	Producer	1.167 (0.189-7.732)	1.169 (0.446-3.097)	0.350 (0.101-1.139)		
			Adjusted odds ratio (95% CI)			
Equol	Non-producer	1.00 (reference)	1.000 (reference)	1.000 (reference)		
	Producer	7.087 (0.173-1021.9)	1.169 (0.311-4.542)	0.052 (0.004-0.361)*		

For calculation of odds ratios refer to Table II legend.

turnover markers also showed no association between ER $\alpha$  polymorphisms and OSI, or between equol production and OSI. When the interactional effect was included as a determinant, it seemed to expose a concealed effect of a difference in ER $\alpha$  polymorphism, and the interactional effect itself reduced the risk with an odds ratio of 0.008. Therefore, a logistic regression analysis was further performed on groups divided by ER $\alpha$  polymorphism (Table III). In the PP allele and Pp allele groups, no statistical difference in equol production was observed in both the crude odds ratio and the adjusted odds ratio. In the pp allele group, although equol production had no statistical difference in crude odds ratio, the adjusted odds ratio showed the low odds ratio of 0.052, the p-value of which was 0.008.

## Discussion

The risk of osteoporosis in elderly women can be assessed by peak bone mass at a young age and the rate of decline in BMD with increasing age because they lack the protective effect of estrogen after menopause. ER seems to play an important role in the maintenance of normal bone turnover, not only before menopause, but also after menopause since phytoestrogens possessing estrogenic bioactivity can be taken in the diet, especially soybeans and soy products. However, humans have ER polymorphisms displaying different affinities to bone turnover. In addition, some are equol producers who can produce highly bioavailable phytoestrogen. Thus, the exact mechanism of the bone-beneficial effect of ER polymorphisms and equol producer/non-producer status has not yet been clarified. In the present study, the effect of equol on bone turnover was analyzed according to ER polymorphisms.

The daily soy intake of the subjects was similar so that the effect of equol production could be assessed regardless of the ER $\alpha$  polymorphism. Univariate analysis showed an interactional effect only in uc-OC, indicating that equol producers with the ER $\alpha$  pp homozygote had higher uc-OC than those with the P allele, whose uc-OC stayed within the normal limits. Since a high level of uc-OC is associated with a high risk of bone fracture (27), multivariate analyses such as logistic regression analysis were applied to exclude the effects of confounding factors.

The crude odds ratio obtained by logistic regression analysis indicated that  $ER\alpha$  polymorphisms and equol production showed no relationship to the decrease in the risk of OSI reduction. However, when odds ratios were adjusted for age, BMI, daily soy intake, and bone turnover markers, an interactional effect of ER $\alpha$  polymorphisms and equol production was recognized as a preventive factor. That is, the odds ratio adjusted according to the ER $\alpha$  polymorphism showed that equol producers with the pp allele group had a decreased risk of OSI reduction. Not only did dietary equol administration to ovariectomized rats show limited bone sparing effects (12), but also long-term consumption of isoflavone-enriched foods did not affect bone metabolism in early postmenopausal women (28). However, these reports did not take ER $\alpha$  polymorphism into account. We showed a relationship between equol production and ERa polymorphisms in bone turnover. That is, equal producers with the ER $\alpha$  pp allele might be protected from bone mineral loss compared to

equol non-producers. The Px haplotype is supposed to be associated with low BMD, but HRT therapy is also reportedly more beneficial for postmenopausal women with a P allele than for those with the pp homozygote (8). In this study, the beneficial effect of equol production on pp homozygotes was observed in subjects including premenopausal women, postmenopausal women, and men. It is likely that the effect of equol production would be more obvious when women with the pp homozygote reach menopause.

The beneficial effects of soybean isoflavone are widely known. A randomized, placebo-controlled clinical trial showed that supplementation of 110 mg aglycones/day is effective to significantly decrease bone resorption in postmenopausal women (29). Daily intake of isoflavones is critical because administration of 60 mg aglycones/day relieves menopausal symptoms, administration between 50 and 110 mg aglycones/ day reduces the risks of breast, colon, and prostate cancer, and administration of 40-60 mg aglycones/day decreases the risk of cardiovascular disease (30). On the other hand, the average intake of soy of ~30 g/day provides nearly 40 mg isoflavones/ day (31). In the present study, the mean soybean intake was ~60 g/day, which was equivalent to ~80 mg/day isoflavone. This consumption might be beneficial for bone health.

In conclusion, this study indicated that equal producers with the ER $\alpha$  pp allele had a decreased risk of OSI reduction. Although this study is cross-sectional, further investigation is needed to assess the long-term effect of isoflavone supplementation on bone turnover in relation to ER polymorphism.

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