

Association between osteoporosis and polymorphisms of the bone estrogen receptor 1, calcitonin receptor genes in Mongolian postmenopausal women

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Abstract

In this study, we have investigated the association between osteoporosis and estrogen receptor 1 (ER1) 397 T>C, and calcitonin receptor (CALCR) 1340 T>C polymorphisms. Genomic DNA was obtained from 104 persons (52 osteoporotic and 52 healthy controls). Genomic DNA was extracted from EDTA-preserved peripheral venous blood of patients and controls and analyzed by PCR-RFLP. As a result, there was no statistically significant difference in the genotype and allele frequencies of patients and controls for ER1 397 T>C and CALCR 1340 T>C polymorphisms. ER1 CC and TC single nucleotides genotypes compared with TT genotypes was found more significantly women with osteoporosis [$p=0.016$; $p=0.0046$, $OR=2.66$; 0.44 , $95\% \text{ CI } 1.185-5.988$; $0.199-0.991$]. There was no statistically significant difference in the genotype and allele frequencies of patient and controls for ER1 combined nucleotides [$p=0.148$, $OR=1.051$, $95\% \text{ CI } (0.993-1.112)$]. Our study showed that CALCR genes single and combined nucleotides genotypes were not significant women with osteoporotic and healthy postmenopausal women.

1. Introduction

Postmenopausal osteoporosis is the most common bone disease, associated with low bone mineral density (BMD) and pathological fractures which lead to significant morbidity. According to the International Osteoporosis Foundation (IOF), 30–50 % of women and 15–30 % of men will be afflicted in the course of their lives. It affects one in three postmenopausal women and the majority of the elderly. The incidence of osteoporotic fractures increases with age and it is higher in whites than blacks, also in Caucasian women (Choi et al., 2012; Gennari et al., 2005; Lee et al., 2010; Ralston, 2010; Ralston and Uitterlinden, 2010). The major determinant of bone strength and osteoporotic fracture risk is bone mineral density (BMD), as assessed by bone ultrasound.

Osteoporosis is classified into many types, but postmenopausal osteoporosis is 80% of all cases. Menopausal changes mainly relate to the decline in the ovarian function and it's hardly controlled under the hypothalamo-pituitary-ovarian axis (Vikhlaev E.M, 1997; Kurz C, 2007). Osteoporosis is now recognized as one of the major problems facing postmenopausal women and osteoporosis induced fracture cause people to become bedridden with serious complication. Osteoporosis is a multifactorial disorder with a strong genetic component. Twin and family studies suggest that about 50–85% of the variance in BMD is genetically determined (Ralston, 2010).

There are several genes that play a role in the genetic determination of osteoporosis. In this regard, a large number of polymorphisms in multiple candidate genes have been investigated (Ralston, 2010). One of the important and widely studied candidate genes for osteoporosis is estrogen receptor 1 (ER1). ER1 gene encodes ligand-activated transcription factor estrogen receptor alpha which belongs to nuclear receptor superfamily. Estrogen

deficiency plays a major role in the pathogenesis of postmenopausal osteoporosis (Ivaska and Kaisa, 2005; Jeedigunta et al., 2010). The skeletal effects of estrogens are mediated by its binding to specific estrogen receptors. While many polymorphisms have been described in ER1, the most widely studied are T397C and C351G polymorphisms located in the first intron. It is noted that the T>C transition associated with loss of the PvuII site results in a potential binding site for *myb* transcription factors. Thus, in some settings, the presence of the T allele might amplify ER1 transcription (Gennari et al., 2005).

The 1340 T>C polymorphism among human calcitonin receptor (CALCR) (also named CTR) gene polymorphism has generated interest precisely because of this single nucleotide polymorphism (SNP) in the coding region. It has been suggested that this locus modulates the susceptibility of postmenopausal women to osteoporotic phenotypes (Lee et al., 2010). CALCR is a member of the transmembrane receptor family and a point mutation polymorphism (1340 T>C) (codon 447) has been identified in the 3-region of the calcitonin receptor gene which included a Pro→Leu shift in the third intracellular domain of the protein (Masi and Brandi, 2007). This change may play a role in G-protein coupling and signal transduction (Wolfe et al., 2003). The absence of the proline residue could alter the secondary structure of the calcitonin receptor (Wolfe et al., 2003).

Correlation of osteoporosis with risk factors and relating genotypes has not been studied among Mongolian postmenopausal women. Therefore aim of our study is to investigate if there is an association between ER1 397 T>C and CALCR 1340 T>C gene polymorphisms and osteoporosis risk in these population.

2. Materials and methods

2.1. Subjects

104 postmenopausal women with a mean age of 50.2 ± 4.5 years were included in the study. Among them, a total of 52 had osteoporosis (T score -2.5 , mean age 51.75 ± 3.28 years) and 52 had normal BMD (T score > -1 , mean age 48.42 ± 3.29 years). Subjects with a history of bone disease, metabolic or endocrine disorders such as hyperthyroidism and hyperparathyroidism, diabetes mellitus, liver disease renal disease, and medications known to affect bone metabolism (e.g., corticosteroids, anticonvulsants, and heparin sodium) were excluded. None of the women had a history of taking medicines for the treatment of osteoporosis, such as active vitamin D3, bisphosphonates, SERM, or calcium. All subjects were from the 4 districts from Ulaanbaatar, Mongolia. The subjects were interviewed using a standard questionnaire including demographics, body mass index (BMI), years since menopause, menarche age, menopause age and history. All the patients gave informed consent and the study was approved by the ethical committee of Health Sciences University of Mongolia.

2.2. Laboratory findings

We took 5 ml fasting blood from venous and separated and kept it frozen until assayed and we measured serum PTH levels by ELISA method and serum calcium, phosphorus and vitamin D level.

2.3 Bone mineral density measurements

The bone ultrasound Mini-Omni (Sunlight, Beammed, Isreal) was used to assess BMD. The forearm and tibia bone scans were performed with the patient on the imaging table using the protocols recommended by the manufacturer. Osteoporosis was defined according to the conventional World Health Organization (WHO) definition.

2.4. DNA extraction and determination of ER1, and CALCR genotypes

Peripheral venous blood samples were obtained from all subjects, and genomic DNA was extracted from EDTA-preserved peripheral venous blood of patients and controls by DNA purification commercial kit (Quagen). Genotyping of ER1 and CALCR polymorphisms were determined by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis. PCR amplification was performed in thermal cycler (Primus, MBG Biotech) and digestion of the PCR products was carried out with restriction enzymes. The primer sequences, restriction enzymes, and fragment lengths are given in (Table 1).

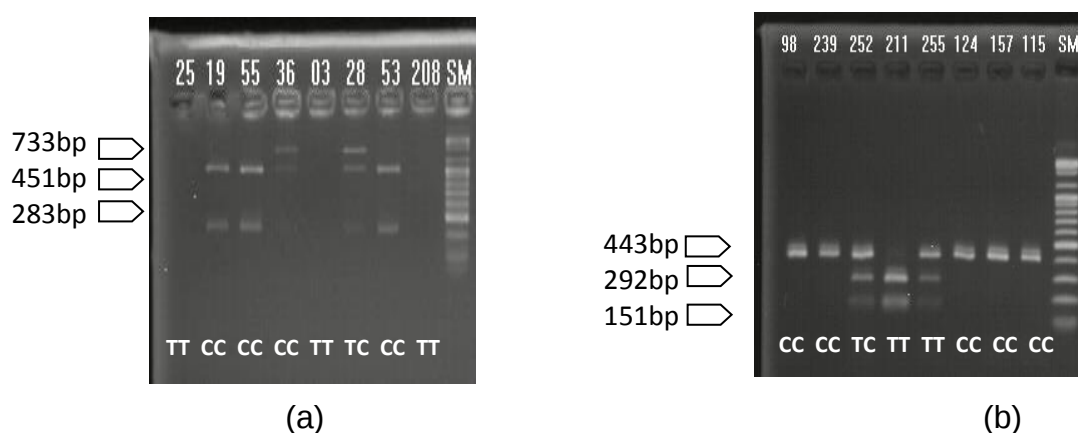
ER1 and CALCR polymorphisms were determined by a slight modification of the polymerase chain reaction (PCR) described by Bandres et al.,(2005), Tural et al., 2012. Amplification of the 1361 bp fragment encompassing the ER1 397 T>C polymorphic site was performed in 25 µl, 1x PCR buffer containing 20 pmol of each primer, 2 mM MgCl₂, 200 µM of each dNTP, 200 ng DNA, and 1.5 U Taq polymerase (Promega). Following initial denaturation at 94 °C for 10 min, amplification was performed by 35 cycles of denaturation at 94 °C for 60 s, annealing at 64 °C for 60 s, and extension at 72 °C for 1.5 min. The reaction was terminated by final extension at 72 °C for 10 min (Bandres et al., 2005). Amplification of the 443 bp fragment encompassing the CALCR 2046 1340 T>C polymorphic site was performed in 25 µl, 1xPCR buffer containing 20 pmol of each primer, 2 mM MgCl₂, 200 µM of each dNTP, 200 ng DNA, and 1.25 U Taq polymerase (Promega) (Dean and Sullivan, 2010). Following initial denaturation at 94 °C for 10 min, amplification was performed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 64 °C for 30 s, and extension at 72 °C for 45 s. The reaction was terminated by final extension at 72 °C for 7 min.

The ER1 and CALCR gene PCR products were digested with PvuII, and AluI restriction enzymes respectively at 37 °C for 16 h. PCR reaction products were separated on a 2–3% agarose gel and RFLP digested products were separated on 3% nu-micropore agarose gel stained with ethidium bromide (Fig. 1).

In order to validate the accuracy and reproducibility of this method, each PCR reaction included internal controls for each genotype. Second PCR was performed to confirm samples which results are not clear. Also, to confirm the accuracy of the genotyping, repeated analysis was performed on randomly selected samples.

Figure 1

Determination of *ER1* and *CALCR* gene polymorphisms by PCR-RFLP. (a) *ER1* genotypes, 25;19;55;36;03;28;53-sample ID number, SM-size marker 100 bp (b) *CALCR* genotypes, 98;239;252;211;255;124;157;115-sample ID number, SM-size marker 100 bp



2.4. Statistical analysis

Analysis of the data was performed using the computer software SPSS 20.0. Continuous data were given as mean±SD (standard deviation) and median (min-max), categorical data were given as frequency (percent). The frequencies of the alleles and genotypes in patients and controls were compared with χ^2 analysis. Odds ratio (OR) and 95% confidence intervals were calculated. The comparisons of two groups were performed by t test. P value smaller than 0.05 (two-tailed) was regarded as statistically significant.

3. Results

Clinical and laboratory findings of the patients and controls were given in (Table 2). The statistically significant differences in terms of age at menopause ($p=0.001$), and number of birth ($p=0.008$), serum Vitamin D ($p=0.031$) between osteoporotic patients and controls groups. But we don't found statistically significant differences of BMI, menarche age, serum calcium and phosphorus, parathyroid hormone level between in the 2 groups.

The results of the genotype and allele frequencies of ER1 397 T>C and CALCR 1340 T>C for osteoporosis patients and control groups are presented in Table 3. Our small sample size might be the reason of disequilibrium. ER1 CC and TC single nucleotide genotypes compared with TT genotypes was found more significantly women with osteoporosis [$p=0.016$; $p=0.0046$, OR=2.66; 0.44, 95% CI 1.185-5.988; 0.199-0.991]. There was no statistically significant difference in the genotype and allele frequencies of patient and controls for ER1 combined nucleotides [$p=0.148$, OR=1.051, 95% CI (0.993–1.112)].

Our study showed that CALCR genes single and combined nucleotide genotypes were not significantly for osteoporotic and healthy postmenopausal women.

4. Discussion

This study was first performed on the detection of osteoporosis factors correlating with genotypes of ER1 and CALCR among Mongolian postmenopausal women. In Asia some researchers investigated association between osteoporosis and polymorphism of genotypes, but in our country didn't have some clinical research data.

The our results showed that, menopause age is significant for osteoporosis, mean age is similar with Japan, Thailand, but one year earlier than Malaysian women. Also number of birth and serum Vitamin D3 level were significantly between osteoporotic patients and controls groups. Our mean level of serum vitamin D3 is lower than among African American women (John F Aloia et al, 2006) and Belgium women (Audrey Neuprez et al 2007). But we are similar with agreement results of serum vitamin D3 level study for 330 England women with osteoporotic fractures. Also we are similar with conclusion about vitamin D3 deficiency caused of sunlight-induced, dietary intake vitamin D level and also season, latitude and chronic disorders (Natalia O Kuchuk et al, 2008).

The results of the present study showed that polymorphic genotypes of ER1 gene are not associated with osteoporosis in combined form but associated in single nucleotide forms. Also we found that, about CALCR gene single and combined nucleotide forms were not significantly among postmenopausal women with osteoporosis. In the present study, there was no statistically significant difference in the genotype and allele frequencies of patients and controls ER1 397 T>C and CALCR 1340 T>C polymorphisms.

However, results of study Tural Sengul et al, 2012 showed that ER1 and CALCR combined genotypes were significantly increased risk of osteoporosis among postmenopausal women. But our results are ER1 single genotype was found more significantly women with osteoporosis but CALCR single and combined genotypes were found no effect against osteoporosis. ER1 gene 397 T>C (also named Pvu II polymorphism) is also associated with preeclampsia, endometriosis, uterine fibroids and tardive dyskinesia in schizophrenia besides osteoporosis (Lai et al., 2002).

In the combined genotype analysis, ER1/CALCR gene TCCC combined genotype was estimated to have protective effects against osteoporosis and ER1/CALCR CCTT combined genotype was a risk factor for osteoporosis. Kobayashi et al. (1996) first demonstrated the association between ER1 gene 397 T>C polymorphism and BMD in Japanese women. A study conducted in Indian women indicated that average lumbar vertebra BMD value of TT genotypes was found to be higher than those of the CC genotype (Mitra et al., 2006). These findings are in not agreement with our results showed that, ER1 gene CC and TC single nucleotide genotypes were more significantly than TT genotype. Our results are also in not agreement with the findings of Erdogan et al (2010) study which was carried out in 126 postmenopausal Turkish women and which revealed that average lumbar vertebra BMD value of women with TT genotype was significantly higher than that with CC genotype. The recent study performed in a south Indian population (Andhra Pradesh et al, 2010) declared that ER1 gene CC allele was significantly higher in pre- and postmenopausal osteoporotic when compared to controls (Jeedigunta et al., 2010). These findings are similar with our results.

A meta-analysis pooled the association between the ER1 polymorphisms and BMD in 16 eligible studies involving 4,297 Chinese women and the data suggested that TT homozygotes had lower BMD than TC/CC genotype (Ralston, 2010). However another meta-analysis (Ioannidis et al., 2002) including eight European centers did not find a significant association between ER1 gene polymorphisms and BMD. Our results found similar findings between ER1 gene and BMD in postmenopausal women.

Calcitonin receptor gene is one of the most important genes for predisposition to osteoporosis. Masi and colleagues first identified an association between CALCR genotype and BMD in a study of Italian women in 1998 and Tsai et al. (2003) reported that women with genotype TT had a greater risk for developing osteoporosis at the lumbar spine and at the femoral neck and this polymorphism is associated with reduced bone mineral density and predisposes women to osteoporosis. Zofkova et al. (2003) suggested that this polymorphism is associated with bone mass at the femoral neck in a cohort of postmenopausal women, the lower values being found in carriers of C allele compared to subjects with the TT genotype. Similarly Bandres et al. (2005) studied 177 postmenopausal Spanish women and revealed that women with the CC genotype had a lower adjusted BMD at the femoral neck. Drews et al. (2005) studied 139 postmenopausal Polish women and they reported T-score was higher in CT genotype carriers. But in the present our study, CALCR gene single and combined genotype was found not any effect against osteoporosis. It means we couldn't find any correlation between CALCR gene and osteoporosis risk in postmenopausal women.

In the combined genotype analysis, ER1/CALCR TCCC combined genotype was found to have a protective effect against osteoporosis. However, ER1/CALCR CCTT combined genotypes were risk factors for osteoporosis. Tural S et al (2012) showed that ER1/CALCR combined genotypes were more significantly among osteoporotic population. But our findings showed that, not correlation between ER1/CALCR genes combined genotypes and osteoporosis.

The effect of an individual SNP is generally small, and the genetic to increased osteoporosis risk. This is the first study to examine the possible relationship between polymorphism of ER1 397 T>C and CALCR 1340 T>C gene polymorphisms and osteoporosis and also to investigate the effect of nucleotide genotype in the Mongolian postmenopausal women. The limitation of our study is the sample size that is relatively small because none of the women had a history of taking medicines for the treatment of osteoporosis.

In conclusion, the polymorphic genotype of ER1 is not found to be associated with osteoporosis in a combined form but found to be associated in single forms. By enlarging the

study we hope that more explanatory and definitive results can be obtained. And further studies are also necessary in different populations.

Conflict of interest

None

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