Gene 515 (2013) 167–172

Contents lists available at SciVerse ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Association between osteoporosis and polymorphisms of the *bone Gla protein*, *estrogen receptor 1*, *collagen 1-A1* and *calcitonin receptor* genes in Turkish postmenopausal women

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ARTICLE INFO

Article history:
Accepted 10 October 2012
Available online 5 November 2012

Keywords:
BGLAP
ER1
COL1A1
CALCR
Polymorphism
Osteoporosis

ABSTRACT

In this study, we have investigated the association between osteoporosis and osteocalcin (*BGLAP*) −298 C>T, estrogen receptor 1 (*ER1*) 397 T>C, collagen type1 alpha 1 (*Col1A1*) 2046 G>T and calcitonin receptor (*CALCR*) 1340 T>C polymorphisms. Genomic DNA was obtained from 266 persons (158 osteoporotic and 108 healthy controls). Genomic DNA was extracted from EDTA-preserved peripheral venous blood of patients and controls by a salting-out method 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 *BGLAP* −298 C>T, *Col1A1* 2046 G>T, *ER1* 397 T>C and *CALCR* 1340 T>C polymorphisms. However, *ER1* CC genotype compared with TT+TC genotypes was found to increase the two fold the risk of osteoporosis [p=0.039, OR=2.156, 95% CI (1.083–4.293)] and *CALCR* CC genotype compared with TT+TC genotypes was found to have protective effect against osteoporosis [p=0.045, OR=0.471, 95% CI (0.237–0.9372)]. In the combined genotype analysis, *ER1*/*CALCR* TCCC combined genotype was estimated to have protective effect against osteoporosis [p=0.0125, OR=0.323, 95% CI (0.1383–0.755)] whereas *BGLAP*/*Col1A1* CCTT and *ER1*/*CALCR* CCTT combined genotypes were estimated as risk factors for osteoporosis in Turkish population (p=0.027, p=0.009 respectively).

1. Introduction

Osteoporosis (OMIM166710) is a common skeletal disorder characterized by low bone mass and microarchitectural deterioration of bone tissue with increased susceptibility to fracture (Ralston and Uitterlinden, 2010). 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 (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 dual photon absorptiometry or dual energy X-ray absorptiometry (DEXA). 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).

Osteocalcin (*BGLAP*) (also known as bone Gla protein, BGP) is one of the major noncollagenous proteins of bone (Chen et al., 2001). Noncollagenous proteins together with collagen, contribute to structural and mechanical properties of bone (Sroga and Vashishth, 2012). *BGLAP* also plays a role in bone resorption and remodeling (Wu et al., 2003). *BGLAP* gene −298 C>T polymorphic promoter region is important for the controlling expression of the *BGLAP* gene (containing osteocalcin box) (Wu et al., 2003). Various promoter elements lying less than a kilobase 5′ to the transcription initiation site contribute to basal expression and osteoblast specificity (Ivaska and Kaisa, 2005). −298 C>T polymorphism in the promoter region has been associated with osteoporosis in postmenopausal women (Chen et al., 2001).

The other important and widely studied candidate gene for osteoporosis is estrogen receptor 1 (*ER1*). *ER1* gene encodes ligand-activated

Abbreviations: *BGLAP*, osteocalcin bone Gla protein; *ER1*, estrogen receptor 1; *Col1A1*, collagen type1 alpha 1; *CALCR: CTR*, calcitonin receptor; *PCR*, polymerase chain reaction; RFLP, restriction fragment length polymorphism; *IOF*, Osteoporosis Foundation; *DEXA*, dual energy X-ray absorptiometry; *SNP*, single nucleotide polymorphism; *BMI*, body mass index; *WHO*, World Health Organization; *SD*, standard deviation; *Cis*, confidence intervals; *OP*, osteoporosis.

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http://dx.doi.org/10.1016/j.gene.2012.10.041

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 *ESR1*, 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 *ESR*1 transcription (Gennari et al., 2005).

Another important candidate gene for predisposition to osteoporosis is the collagen type1 alpha 1 (*COL1A1*) gene, which encodes the 1(I) protein chain of type I collagen. It makes up 90% of the organic matrix that has a role in bone mineralization and gives flexibility to the bone (Erdogan et al., 2010). It is a heterotrimer protein consisting of two α1 chains and one α2 chain of type 1 collagen. The Sp1 transcription factor binding site is located in the first intron of the *COL1A1* gene, an important region for the regulation of collagen transcription (Hubacek et al., 2006). This is a single nucleotide polymorphism affecting the recognition site of the transcription factor Sp1. Presence of the T allele leads to abnormal production of the α-1collagen chain in comparison to the α-2 collagen chain, which has an adverse effect on bone composition and mechanical strength (Hubacek et al., 2006).

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).

The aim of this study is to investigate if there is an association between *BGLAP* −298 C>T, *ESR1* 397 T>C, *Col1A1* 2046 G>T and *CALCR* 1340 T>C gene polymorphisms and osteoporosis risk in a Turkish population.

2. Materials and methods

2.1. Subjects

266 postmenopausal women with a mean age of 61.60 ± 8.51 years were included in the study. Among them, a total of 158 had osteoporosis (T score< −2.5, mean age 63.65 ± 8.62 years) and 108 had normal BMD (T score> −1, mean age 58.81 ± 7.97 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 Black Sea Coastal region from Turkey. 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 Ondokuz Mayıs University.

2.2. Bone mineral density measurements

Dual-energy X-ray absorptiometry (Norland EXCELL, USA) was used to assess BMD. Left hip and posterior-anterior lumbar spine (L2–L3–L4) scans were performed with the patient lying supine on the imaging table using the protocols recommended by the manufacturer. Osteoporosis was defined according to the conventional World Health Organization (WHO) definition.

2.3. DNA extraction and determination of BGLAP, ER1, Col1A1 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 a salting-out method (Miller et al., 1988). Genotyping of *BGLAP*, *ER1*, *Col1A1* and *CALCR* polymorphisms were determined by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis. PCR amplification was performed in thermal cycler (Techne Gradient, Cambridge, UK) 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.

Osteocalcin and *Col1A1* genes polymorphism identification were conducted by a slight modification based on the method of Chen et al. (2001) and Todhunter et al. (2005) respectively. *ER1* and *CALCR* polymorphisms were determined by a slight modification of the polymerase chain reaction (PCR) described by Bandres et al. (2005).

Amplification of the 253 bp fragment encompassing the *BGLAP −298 C>T* polymorphic site was performed in 25 μl, 1× PCR buffer containing 10 pmol of each primer (GmbH Biotech, Deutschland), 2 mM MgCl2, 200 μM of each dNTP (MBI, Fermentas, Lithuania), 200 ng DNA, and 1.5 U Taq polymerase (Promega, Madison, WI, USA). After initial denaturation at 94 °C for 8 min, amplification was performed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 64 °C for 30 s, and extension at 72 °C for 60 s. Final extension was allowed to proceed at 72 °C for 10 min (Chen et al., 2001).

Amplification of the 1361 bp fragment encompassing the *ER1* 397 T>C polymorphic site was performed in 25 μl, 1× 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

Table 1
Single nucleotide polymorphisms (SNPs) investigated in this study and PCR primers for the genotyping of *BGLAP*, *ER1*, *COL1A1* and *CALCR*.

Gene and polymorphism	Region	rs number	Primer pairs	Method	Genotype			
					Wild Ref	Heterozygote		Variant
BGLAP (−298C>T)	Promoter	rs1800247	(F)5′CCGCAGCTCCCAACCACAATAAGCT-3′[a] (R)5′CAATAGGGCGAGGAGT-3′	*Hind* III based PCR-RFLP	CC	CT	TT	[5]
ER1 (+397 T>C)	Intron 1	rs2234693	(F)5′CTGCCACCCTATCTGTATCTTT-3 (R)5-ACCCTGGCGTCGATTATCTGA-3′	*Pvu* II based PCR-RFLP	TT	TC	CC	[16]
Col1A1 (+2046 G>T)	Intron 1	rs1800012	(F)5′CTGGACTATTTGCGGACTTTTTGG-3′ (R)5′GTCCAGCCCTCATCCTGGCC-3′	*MscI*/*Bal* I based PCR-RFLP	GG	GT	TT	[15]
CALCR (+1377 T>C)	Exon 13	rs1801197	(F)5′AGGTCCAAACCACCGTGAAG-3′ (R)5′GCAGTGGGAGACTCCATTCC-3′	*Alu* I based PCR-RFLP	TT	TC	CC	[16]

[a] F: forward primer, R: reverse primer.