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Investigation of Association Between TLR4 Gene Polymorphisms and Osteoporosis in Postmenauposal Turkish Women

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ABSTRACT

Background: Postmenopausal osteoporosis is a systemic bone disease that is characterized by accelerated bone loss after menopause and an increased risk of fractures. The immune system and cytokines regulate bone metabolism. Toll-like receptors (TLRs) play an important role in the induction and regulation of the innate immune system and adaptive immune responses.

Objectives: To investigate the association between TLR4 gene polymorphisms and bone mineral density (BMD) in postmenopausal osteoporotic (OP) and nonosteoporotic (NOP) Turkish women.

Patients and Methods: The study population consisted of 178 OP and 178 NOP Turkish women. BMDs were obtained by dual-energy X-ray absorptiometry. Two single-nucleotide polymorphisms (SNPs) of the TLR4 gene (Asp299Gly and Thr399Ile) were examined by polymerase chain reaction-restriction fragment length polymorphism. The frequency of polymorphisms and the possible relationships between genotypes and BMD were the main outcome measures.

Results: Lumbar BMD of OP women was significantly lower than in NOP women ($P = 0.04$), but total hip BMD and Z scores did not differ. The frequency of the Asp299Gly and Thr399Ile polymorphisms in our population was 18% and 15%, respectively. There was no significant difference in the frequency of TLR4 gene (Asp299Gly and Thr399Ile) polymorphisms between OP and NOP women, but carriers of heterozygous genotypes had lower BMDs ($P < 0.01$ and $P < 0.01$).

Conclusions: We observed lower BMDs in carriers of heterozygous genotypes of polymorphisms than homozygous mutant genotypes. With our limited population, no firm conclusions can be drawn as to what extent low bone mineral density is associated with these heterozygous genotypes, and further studies are needed to analyze our results.

Implication for health policy/practice/research/medical education:
This study gives a new vision about genetic basis of osteoporosis which has multifactorial etiology. New pharmacological approaches could be improved for treatment and prevention of osteoporosis.

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1. Background

Postmenopausal osteoporosis is a systemic bone disease that is characterized by accelerated bone loss after menopause and increased risk of fractures. Estrogen deficiency during menopause is one of the major underlying factors of osteoporosis in women (1). Estrogen deficiency is associated with accelerated osteoblast

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apoptosis and increased production of pro-inflammatory cytokines (2). Exposure of bone cultures to supernatants from activated leukocytes is associated with induced osteoclast formation (2-4). In the molecular pathogenesis of osteoporosis, inflammatory cytokines play a key role in osteoclast interactions during bone turnover (4). The relationship between bone turnover and inflammatory cytokines, their receptors, and intracellular signaling pathways has been recently investigated with regard to osteoporosis (5-8).

Toll-like receptors (TLRs) are pattern recognition receptors (PRRs) and initiate cellular signaling pathways to activate immune response genes, including inflammatory cytokines (9). Ten human TLRs have been identified (5). Each TLR recognizes specific ligands, including lipoproteins, lipoteichoic acid, and zymosan by TLR2; dsRNA by TLR3; lipopolysaccharide (LPS) by TLR4; flagellin by TLR5; ssRNA by TLR7/8; and CpG DNA by TLR9. Twenty-nine single-nucleotide polymorphisms (SNPs) have been identified in the human TLR4 gene (10).

TLR4, a member of the TLR family, is expressed on cardiomyocytes, macrophages, airway epithelium, and endothelial and smooth muscle cells (11, 12) and recognizes endogenous ligands, such as fibronectin and several heat shock proteins (HSPs). Two nonsynonymous and common gene polymorphisms in the extracellular domain of TLR4, Asp299Gly and Thr399Ile, have been suggested to alter its function and cause hyporesponsiveness to LPS in human alveolar macrophages and airway epithelial cells (12). Individuals who carry the Asp299Gly allele of TLR4 have lower levels of proinflammatory cytokines, acute-phase reactants, and soluble adhesion molecules, such as interleukin 6 and fibrinogen (13). The reduced but chronic expression of these cytokines causes chronic inflammation, which leads to the transformation of macrophages into osteoclasts over long periods (14).

The osteoclast/osteoblast balance is a distinctive marker of bone turnover and osteoporosis. Postmenopausal osteoporosis is a multifactorial condition that we examined with regard to the possible interaction between BMD and TLR4 gene polymorphisms in postmenopausal Turkish women.

2. Patients and Methods

2.1. Study Population

We conducted a multicenter, prospective, controlled study with Firat University Hospital, Elazig and Kecioren Education and Research Hospital, Ankara, Turkey. The protocol of this study was approved by the ethics committee of Firat University Hospital. A total of 356 postmenopausal women—178 osteoporotic (OP) (T score on BMD measurement < -2.5 SD) and 178 nonosteoporotic women (NOP) (T score on BMD measurement > -1.0 SD) —who visited the menopause out-patient clinic of Keciören Education and Research Hospital and Nuclear Medicine Clinic of Firat University Hospital between May 2009 and November 2009 were included in our study. Demographics and lifestyle factors, including menopausal period, smoking history, average daily alcohol consumption, and dietary calcium intake, were recorded through lifestyle and food frequency questionnaires that were completed at baseline. Body weight and height were measured at baseline, and body mass index (BMI) was calculated. A detailed medical history of the subjects was obtained through health questionnaires. Blood fasting glucose and hepatic and renal functions were measured. Women who had undergone an ovariectomy or who had hepatic or renal disease, diabetes mellitus, or other endocrine diseases were excluded from this study. None of the subjects had received any medication that is known to affect bone metabolism (such as glucocorticoids, thyroxin, antiepileptics, bisphosphonates, calcitonin, and hormone replacement therapy for more than 3 months). Those who had conditions that were known to affect BMD were excluded from the study. All interviews were conducted by trained interviewers. Informed consent was obtained from all subjects at the time of blood sampling.

2.2. BMD Measurement

Areal BMD (g/cm2) at L2–L4 of the lumbar spine and total hip was measured by dual energy X-ray absorptiometry (DEXA). Densitometers were calibrated daily. The coefficient of variation for DEXA instrumentation was 0.52%. OP and NOP groups consisted of women whose T scores at the lumbar spine by DEXA were lower than -2.5 and higher than -1, respectively.

2.3. Genotyping

Genomic DNA from patients and controls was extracted from peripheral white blood cells using the Wizard Genomic DNA Extraction Kit (Promega, USA), per the manufacturer's instructions.

2.4. PCR

Two polymorphisms of the TLR4 gene (Asp299Gly and Thr399Ile) were examined in 178 OP and 178 NOP women using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Detection of TLR4 polymorphisms was performed per Lorenz et al. (15). The PCR primers for Asp299Gly and Thr399Ile were as follows:

TLR4Asp299Gly Forward: 5'GATTAGCATACTTAGACTACTACCTCCATG

Reverse: 5'GATCAACTTCTGAAAAAGCATTCCCAC

TLR4 Thr399Ile Forward: 5'GGTTGCTGTTCTCAAAGTGATTTTGGGAGAA

Reverse: 5'CCTGAAGACTGGAGAGTGAGTTAAATGCT

The program comprised 30 cycles of the following: 95°C for 4 min, 95°C for 30 s, 55°C for 30 s, and 72°C for

30 s. The amplicons were cleaved with NcoI (Asp299Gly) and HinfI (Thr399Ile), respectively. Digested products were separated on a 3% agarose gel.

2.5. Statistical Analysis

Statistical analysis was performed using SPSS 12.0 (Inc., Chicago, Illinois, USA). Results were expressed as mean and standard deviation or number and percentage, as appropriate. Differences between means were analyzed by student's t-test and Mann-Whitney U-test according to the distribution of data. The significance of differences between groups was assessed by chi-squared test or Fisher's exact test for categorical variables, where applicable. Hardy-Weinberg equilibrium was tested for each genotyped SNP using $\chi 2$ statistics. Stepwise regression was used to analyze lumbar spine and total hip BMD, using BMI, age, smoking, and alcohol intake to identify significant covariates of BMD. ANOVA was performed to analyze the lumbar spine and total hip BMD of each SNP genotype. P values < 0.05 were considered significant for all analyses.

3. Results

This study was designed to detect any association between BMD and the Asp299Gly and Thr399Ile TLR4 polymorphisms in postmenopausal OP and NOP Turkish women. For both groups, the menopausal period was at least 5 years. The demographics of the women are presented in *Table 1*. BMI and age were covariates that approached statistical significance on fitting BMD in the regression model. Genotype frequencies of Asp299Gly for OP and NOP women are shown in Table 2. The allelic frequencies of Asp299Gly in OP women were 87% A allele and 13% G allele versus 90% A allele and 10% G allele in NOP women.

The genotype frequencies of Thr399Ile for the groups are shown in Table 2. The allele frequencies of this polymorphism in OP and NOP women were 88% C allele and 12% T allele versus 93% C allele and 7% T allele, respectively (*Table 2*). We did not detect any significant difference in genotype or allele distribution for the 2 polymorphisms between groups. Homozygous mutant genotypes of these polymorphisms were rare in our small population. The frequency of alleles approximated Hardy-Weinberg equilibrium ($\chi 2 = 2.4$ $P = 0.6$).

We examined the difference in lumbar spine and total hip BMD between genotypes (*Table 3*). The heterozygous genotypes (AG and CT) had significantly lower BMDs than wild-type genotypes. There was no significant difference in BMI between genotypes in the entire study population (*Table 3*).

Table 1. Demographic Characteristics of All Women in the Study

Characteristics [b]	OP [a] (n : 178)	NOP [a] (n : 178)	*P* value
Age, y	57 ± 7	57 ± 6	0.89
Weight, kg	70 ± 13	71 ± 14	0.39
Height, cm	156 ± 6	154 ± 7	< 0.01
BMI [a], kg/m^2	29 ± 5	30 ± 5	0.02
Lumbar spine BMD [a], g/cm^2	0.969 ± 0.199	1.023 ± 0.278	0.04
Total hip BMD, g/cm^2	0.977 ± 0.146	1.008 ± 0.209	0.11
Lumbar spine Z score	-0.7 ± 1.5	0.5 ± 2.0	0.26
Total hip Z score	-0.3 ± 1.2	-0.1 ± 1.5	0.18

[a] Abbrevietions: OP, Osteoporotic group; NOP, Nonosteoporotic group; BMI, Body mass index; BMD, Bone mineral density
[b] Values are expressed as Mean ± SD

Table 2. Genotype and Allele Frequencies of All Women in the Study

	OP [a] No,%	NOP [a] No, %	*P* value
Asp299Gly Genotypes			
AA (Asp/Asp)	139 (78)	145 (82)	> 0.05
AG (Asp/Gly)	33 (19)	31 (17)	> 0.05
GG (Gly/Gly)	6 (3)	2 (1)	> 0.05
Total	178 (100)	178 (100)	> 0.05
Asp299Gly Alleles			
A (wild allele)	311 (87)	321 (90)	> 0.05
G (mutant allele)	45 (13)	35 (10)	> 0.05
Total	356	356	> 0.05
Thr399Ile Genotypes			
CC (Thr/Thr)	140 (79)	154 (87)	> 0.05
TT (Ile/Ile)	6 (3)	2 (1)	> 0.05
Total	178 (100)	178 (100)	> 0.05
Thr399Ile Alleles			
C (wild allele)	312 (88)	330 (93)	> 0.05
T (mutant allele)	44 (12)	26 (7)	> 0.05
Total	356 (100)	356 (100)	> 0.05

[a] Abbrevietions: OP, Osteoporotic Group; NOP, Nonosteoporotic Group

4. Discussion

Postmenopausal osteoporosis is a multifactorial systemic bone disease, and estrogen deficiency is one of the major underlying factors of osteoporosis in women (1). Human and animal experiments have implicated inflammatory cytokines as primary mediators of the accelerated bone loss during menopause (16). Estrogen deficiency is associated with increased production of pro-inflammatory cytokines, and exposure of bone cultures to supernatants from activated leukocytes is associated with induced osteoclast formation (2-4). Further, in bone, estrogen deficiency is linked to accelerated osteoblast apoptosis and predisposition to osteoporotic fractures (17). The most frequently examined genetic topics of osteoporosis are the immune system, inflammatory cytokines, and their receptors and the mechanism by which osteoblastic and osteoclastic activity is regulated (16-18). TLR4 may participate in the development of osteoporosis, in addition to its well-known function in the immune response, by recognizing endogenous ligands, such as fibronectin and heat shock proteins (HSPs). Many studies in Caucasians suggest that the TLR4 polymorphism Asp299Gly is as-

Table 3. Lumbar Spine and Total Hip BMD and BMI of Each Genotype for All Women in the Study

	Asp299Gly Genotype [b]					
	Lumbar spine BMD [a] (g/cm^2)	*P* value	Total hip BMD (g/cm^2)	*P* value	BMI [a] (kg/m^2)	*P* value
Asp/Asp (AA)	1.015 ± 0.251	0.010	1.005 ± 0.188	0.019	30 ± 5	0.31
Asp/Gly (AG)	0.914± 0.176	0.010	0.935 ± 0.125	0.019	29 ± 4	0.31
Gly/Gly (GG)	0.962 ± 0.296	0.010	1.002 ± 0.216	0.019	28 ± 2	0.31
	Thr399Ile Genotype [b]					
	Lumbar spine BMD [a] (g/cm^2)	*P* value	Total hip BMD (g/cm^2)	P value	BMI [a] (kg/m^2)	*P* value
Thr/Thr (CC)	1.012 ± 0.250	0.019	1.006 ± 0.186	0.007	30 ± 5	0.21
Thr/Ile (CT)	0.912 ± 0.166	0.019	0.922 ± 0.125	0.007	28 ± 5	0.21
Ile/Ile (TT)	0.963 ± 0.296	0.019	1.003 ± 0.216	0.007	28 ± 2	0.21

[a] Abbreviations: BMI, Body mass index; BMD, Bone mineral density
[b] Values are expressed as Mean ± SD

sociated with innate immunity-related diseases, such as chronic inflammatory disease and atherosclerosis (13, 19, 20), but in the Asian population, this polymorphism is rare (21, 22). Kim *et al*. detected the Asp299Gly or Thr399Ile mutations in any of the 225 diabetic and 153 healthy Korean subjects, and the frequency of TLR4 polymorphisms differed between ethnicities in the Asian population; the prevalence of osteoporosis was not discussed in this population (23). In our study, the frequency of both TLR4 polymorphisms was 15% and 18% in the Turkish population. We observed a relationship between bone mineral density and heterozygous genotypes of the Asp299Gly and Thr399Ile TLR4 polymorphisms. Women who were carriers of heterozygous genotypes had significantly lower lumbar spine and total hip BMDs than those with homozygous genotypes. We suggest that TLR4 polymorphisms and their effects on gene expression might change bone turn-over against bone formation [BPS1]. Induced macrophage and osteoclast formation by a chronic low-dose inflammatory microenvironment as a result of these polymorphisms in bone tissue might trigger bone loss. We expected to detect a more pronounced effect on bone loss by homozygous mutant genotypes, but we observed higher bone mass in this small population.

In the literature, no study except Santos *et al*. has examined TLR4 polymorphisms and BMD; thus, we will discuss our theory on the basis of inflammation. Santos *et al*. reported frequencies of the Asp299Gly and Thr399Ile TLR4 polymorphisms in 227 Chilean elderly women of 4.6% and 4.4%, respectively, and any association between TLR4 Asp299Gly and bone mineral density was reported for elderly Chilean women; they discussed their results with regard to inflammation, not peak bone mass (24).

Kim *et al*. reported that HSP60 was higher in postmenopausal women than premenopausal women and that HSP60 significantly reduced osteoblast viability through increased expression of TLR-2 and TLR-4. Also, they found that blocking antibodies to TLR-2 and TLR-4 eliminated the effects of HSP60 on apoptosis. HSP60 upregulated the expression of TLR-2 in bone marrow-derived macrophages, and pretreatment with a TLR-2-blocking antibody inhibited HSP60 and cytokine-induced potentiation of osteoclast formation and bone resorption (25). TLR4 and TLR2 become activated through reactive oxygen species and HSPs, and other non-LPS-dependent mechanisms may effect different signatures of genes and proinflammatory mediators (26).

Johnson *et al*. reported that mutant TLR4 mice have greater bone mineral content ($P < 0.001$) and larger bones ($P < 0.001$, as measured by bone area) than wild-type mice with the same genetic background—differences that increased with age. The mutant mice (loss of TLR4 function) had significantly higher bone mineral density ($P < 0.001$), and this difference increased with age ($P < 0.001$) (27). TLR4 has been believed to function only in inflammation and immunity, profoundly affecting the body shape—effects that increase with age. How mutations in TLR4 effect changes in bone and fat is not known, but bone growth might be regulated by TLR4 on osteoblasts, osteoclasts, or stromal cells—the common precursors of osteoblasts and adipocytes (28).

The Thr399Ile TLR4 polymorphisms cosegregates with Asp299Gly (29). We observed that an allelic mutation of TLR4 gene by TLR4 Asp299Gly and Thr399Ile polymorphisms was related to lower bone mass and that mutant homozygotes had higher bone mass than heterozygotes. Our results are in contrast with Johnson *et al*., and we hypothesize that the chronic inflammatory microenvironment, as a result of these polymorphisms during menopause, shift macrophage transformation to osteoclastic differentiation, increase the response to inflammatory cytokines in bone tissue, or induce osteoblast apoptosis. We can not examine or discuss the results of mutant homozygotes due to their low frequency in our restricted population. Expanded and different population studies are needed to analyze our results.

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