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Toll-Like Receptor 4 Gene Polymorphism C1196T in Polish Women with Postmenopausal Osteoporosis – Preliminary Investigation*

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation;
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Abstract

Background. Postmenopausal osteoporosis is a systemic bone disease characterized by low bone mass after menopause. Bone remodeling is regulated by a number of factors, including the immune system. Toll-like receptors 4 (TLR4) are expressed on bone cells and modify the immune response. TLR4 gene polymorphism may take part in the development of chronic inflammation in women after menopause, which is the cause of severe bone resorption.

Objectives. To examine the frequency of TLR4 C1196T genotypes in postmenopausal osteoporotic and non-osteoporotic Polish women and to investigate the possible relationship between C1196T polymorphism, bone mineral density (BMD) and the incidence of osteoporotic fractures in this group of patients.

Material and Methods. The study involved 40 postmenopausal women with osteoporosis and 63 healthy postmenopausal non-osteoporotic women. BMD measurements were performed by dual-energy X-ray absorptiometry. DNA was extracted from peripheral blood. Genotyping was performed by real-time PCR using LightSNiP tests with SimpleProbe probes. Melting curve analysis of PCR amplicons enabled the identification of individual C1196T genotypes.

Results. C1196T genotype frequencies in the osteoporotic group were 88% for CC and 12% for CT. In the control group, respectively 86% and 14%. We did not observe the TT genotype. There was no association of C1196T genotypes and BMD nor the incidence of fractures but there was a correlation between genotypes and body height ($p = 0.035$, $r = 0.415$). Homozygous subjects for the C-allele had a lower body height with respect to heterozygous subjects.

Conclusions. It is unlikely that TLR4 C1196T polymorphism is related to bone mineral density and fracture incidence in Polish osteoporotic women after menopause. However, our data suggests that the C allele may be associated with lower body height in this group. Due to the small number of participants, our observations should be considered as preliminary. Larger studies are needed to confirm our findings (*Adv Clin Exp Med* 2015, 24, 2, 239–243).

Key words: polymorphism, gene, bone mineral density, postmenopausal osteoporosis, TLR4.

Postmenopausal osteoporosis is a systemic bone disease that is characterized by low bone mass and microarchitectural deterioration of bone

tissue after menopause. This disease has become one of the most important public health problems in our ageing society due to the medical,

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psychological and economic consequences. Genetic and hereditary factors, hormonal status, dietary habits and lifestyle may affect the development of postmenopausal osteoporosis [1–3]. A key role in the development of the disease is played by estrogen deficiency and increased production of pro-inflammatory cytokines. The low estrogen level after menopause is associated with increased osteoblast (bone-forming cell) apoptosis and the production of pro-inflammatory cytokines. These factors are considered strong stimulators of osteoclast activity (bone-resorbing cells) [4–8].

One of the modulators of bone cell function is toll-like receptors (TLRs). TLR are pattern recognition receptors (PRRs) which are expressed on a variety of cell types, including bone cells [9]. TLR4, a member of the TLR family, is one of the best known TLRs. TLR4 recognizes both endo- and exogenous ligands, including lipopolysaccharide (LPS), fibronectin, and several heat shock proteins. It has been shown that TLR4 signaling regulates immune response and bone metabolism [10]. TLR4 expression on osteoclasts depends on the maturity of these cells. In the early precursor cells, these receptors inhibit osteoclastogenesis, whereas in mature osteoclasts they promote this process. TLR-signaling pathways activate immune response genes, including those responsible for pro-inflammatory cytokines. Low but chronic expression of these cytokines causes inflammation and increased bone resorption [12–14].

Deregulation of the TLR4 pathway might result in chronic inflammation and may be a potential risk factor for many immunological diseases. Changes in TLR4 signaling are likely to be linked to TLR4 gene polymorphism. Two polymorphisms in the extracellular domain of TLR4, C1196T (Thr399Ile, rs4986791) and A896G (Asp299Gly, rs4986790), have been suggested to alter its function. C1196T polymorphism is associated with the transition of T instead of C at position base 1196 from the start codon and leads to an exchange of isoleucine instead of threonine in the extracellular domain of TLR4. A896G is associated with a transition of G instead of A at position base 896 and leads to an exchange of glycine instead of aspartic acid. C1196T and A896G are called “loss-of-function” polymorphisms which alter ligand binding to the TLR4 [14, 15]. Structural and functional changes in the TLR4 which are associated with TLR4 gene polymorphism may affect the pathogenesis and course of bone diseases. Despite extensive data on the genetic background of postmenopausal osteoporosis, there are many divergences. The identification of molecular markers linked to this disease makes possible early prevention and effective therapy.

The aim of our study was to examine the frequency of TLR4 C1196T genotypes in postmenopausal osteoporotic and non-osteoporotic Polish women and investigate the possible association of C1196T polymorphism of the TLR4 gene with bone mineral density (BMD) and the incidence of osteoporotic hip, spinal and peripheral fractures in this group.

Material and Methods

Subjects

The study involved 40 postmenopausal women with osteoporosis. The characteristics of the study group are presented in Table 1.

The control group consisted of 63 healthy anonymous postmenopausal women without osteoporosis. Lifestyle factors, including menopausal period, smoking history and dietary calcium intake, were recorded. A detailed medical history of the subjects was obtained. Participants provided their written informed consent to participate in this study. The study was in compliance with the Helsinki Convention and was approved by the local ethics committee.

BMD measurements were performed by dual-energy X-ray absorptiometry (Hologic Delphi machine) at the lumbar spine (L1–L4) and proximal femur (total hip, femoral neck). Results were recorded as T-scores.

Measurement of growth was taken by PROSTAND stadiometer Seca 210.

Weight was measured with a balance beam scale and body mass index (BMI) was calculated.

Table 1. Characteristics of postmenopausal osteoporotic group. Values are expressed as min ÷ max, mean ± SD and percentage

Subjects	40
Age (years)	51 ÷ 85 (73.49 ± 9.33)
Height (cm)	142.0 ÷ 174.0 (154.74 ± 6.45)
BMI (kg/m ²)	17.4 ÷ 31.3 (25.3 ± 3.40)
Neck BMD (g/cm ²)	0.473 ÷ 0.760 (0.581 ± 0.074)
Neck T score	–0.30 ÷ –2.6 (–2.89 ± 0.199)
Spinal fractures	21/40 (53%)
Hip fractures	3/40 (8%)
Peripheral fractures	17/40 (43%)
All types of osteoporotic fractures	31/40 (78%)

BMD – bone mineral density, BMI – body mass index.

Fracture assessment was conducted by gathering information on the history of parental spine, hip and peripheral fractures.

DNA Isolation and Genotyping

Genomic DNA was extracted from peripheral blood samples by standard procedures using the Blood Mini kit (A&A Biotechnology, Poland). TLR4 C1196T genotyping was performed by LightCycler real-time PCR (Roche, Germany) using commercial LightSNiP (SimpleProbe) assays from TIB-MolBiol (Germany), according to the manufacturer's recommendations. Melting curve analysis of the PCR amplicons enabled identification of individual genotypes of the TLR4 gene. The genotypes were classified as homozygote major allele (CC), heterozygote (CT) and homozygote minor allele (TT).

Statistical Analyses

Statistical analyses were performed using STATISTICA 10.0 (StatSoft Inc). To compare the frequency of genotypes and alleles of TLR4 C1196T polymorphism in postmenopausal women with osteoporosis and the control group, the Freeman-Halton extension of Fisher's exact test for contingency table was used. Correlation analysis of C1196T genotypes with body height, bone mineral density, body mass index and osteoporotic hip, spinal and peripheral fractures was performed using Spearman's Rank Correlation Test. Genotype frequencies were tested for their concordance with Hardy-Weinberg equilibrium (HWE) χ^2 ratio. Differences were considered statistically significant at p value less than 0.05.

Results

Our study was performed to examine the frequency of genotypes and alleles of C1196T polymorphism in the TLR4 gene in postmenopausal

osteoporotic and non-osteoporotic Polish women and to investigate the association of C1196T polymorphism and bone mineral density and incidence of osteoporotic fractures. The genotype and allelic frequencies of C1196T in the patients and healthy controls are presented in Table 2.

The distribution of C1196T genotypes was consistent with the Hardy-Weinberg equilibrium in the 2 groups ($\chi^2 = 0.152$; $p = 0.697$ and $\chi^2 = 0.373$; $p = 0.541$, respectively). The genotype frequencies in postmenopausal osteoporotic women were 88% for the CC genotype and 12% for the CT genotype. In the control group, 86% and 14%, respectively. In the 2 groups we did not observe the TT genotype.

We did not find a statistically significant association between C1196T genotypes and neck BMD, neck T score and fracture incidence in postmenopausal osteoporotic women (Table 3 and 4). However, we have identified a statistically significant (at $p = 0.035$, $r = 0.415$) correlation between C1196 genotypes and body height. Homozygous subjects for the C allele had a lower body height compared to heterozygous subjects (Table 4).

Discussion

It is well known that the immune and skeletal systems interact and affect each other [10, 12, 14, 16–19]. Osteoclasts are derived from the same myeloid precursor cells that give rise to macrophages and myeloid dendritic cells. Osteoblasts regulate hematopoietic stem cell niches from which all blood and immune cells are derived. Soluble mediators of immune cells (i.e. cytokines) regulate the activity of bone cells [17]. In the past few years, the most frequently studied topics of osteoporosis are the immune system, pro-inflammatory cytokines with their receptors and immunological mechanisms regulating bone metabolism. TLR4 are expressed on osteoblasts and osteoclasts surface and are important regulators of immune response, and therefore may take part in the development of low grade chronic inflammation in women after

Table 2. TLR4 C1196T genotype and allele frequencies of postmenopausal osteoporotic women and control group

	C1196T genotypes			Alleles	
	CC (wt)	CT	TT (mt)	C	T
Osteoporotic group no. (%)	35 (88)	5 (12)	0 (0)	75 (94)	5 (6)
Controls no. (%)	54 (86)	9 (14)	0 (0)	117 (93)	9 (7)
P-value	> 0.05			> 0.05	

mt – mutant, wt – wild-type.

Table 3. Osteoporotic fractures of each C1196T genotype for postmenopausal osteoporotic women in the study

	C1196T genotypes		P-value
	CC	CT	
Spinal fractures	18	3	> 0.05
Lack of spinal fractures	17	2	
Hip fractures	3	0	> 0.05
Lack of hip fractures	32	5	
Peripheral fractures	14	3	> 0.05
Lack of peripheral fractures	21	2	
All types of fractures	26	5	> 0.05
Lack of fractures	9	0	

Fisher's exact test for 2×2 contingency table.

Table 4. Spearman's rank correlation calculated between TLR4 C1196T genotypes and body height, neck bone mineral density, neck T score and osteoporotic fracture incidence in postmenopausal women with osteoporosis

	C1196T genotypes ^a
Height (cm)	$p = 0.035$; $r = 0.415$
Neck BMD ^b (g/cm ²)	$p > 0.05$
Neck T score	$p > 0.05$
Number of osteoporotic fractures	$p > 0.05$

0 – CC genotype; 1 – CT genotype; BMD – bone mineral density; r – Spearman's rank correlation coefficient.

menopause, which is the cause of severe bone resorption and the development of postmenopausal osteoporosis. Some studies have suggested the role of TLR4 polymorphism in the pathogenesis of immunity-related diseases, such as type 2 diabetes, cardiovascular diseases, cancer, asthma and allergies, infectious diseases and systemic lupus erythematosus [20–26].

This is the first study evaluating the association of C1196T polymorphism of the TLR4 gene

and bone mineral density and osteoporotic fracture incidence in Polish postmenopausal women with osteoporosis. In 2004, Johnson et al. showed the association of C1196T and A896G polymorphisms of the TLR4 gene with higher bone mass in an animal model [27]. So far, in humans only two similar studies have been conducted. In 2006, Santos et al. did not find an association of TLR4 polymorphisms and bone mineral density in elderly Chilean women [28]. In 2012, Ozkan et al. presented the results of their research in the Turkish population. There was no difference in the frequency of TLR4 genotypes in osteoporotic and non-osteoporotic postmenopausal women. However, it was shown that heterozygous CT subjects have a lower BMD compared to homozygous TT mutant subjects [29]. Our results are partly consistent with the findings of Ozkan et al. There was no difference in the C1196T genotype distribution in controls and osteoporotic women. However, we did not find an association between BMD and C1196T genotypes. Moreover, there were no observed associations between C1196T genotypes and incidence of hip, peripheral and all osteoporotic fractures. We have only demonstrated a correlation between body height and C1196T polymorphism of the TLR4 gene. The CC genotype was related to a lower body height compared to heterozygous CT subjects.

Evidence from our study suggests that C1196T polymorphism in the TLR4 gene is not related to bone mineral density and osteoporotic fracture incidence in Polish osteoporotic women after menopause. However, our data suggests that the C allele may be associated with lower body height in this group. The main limitation of our study is a relatively small number of participants, self-reported fractures and low frequency of CT genotype. Therefore our observations should be considered as preliminary. Further studies on larger cohorts of individuals are thus urgently needed to shed more light on the value of assaying the C1196T TLR4 polymorphism in postmenopausal women. Assessment of these associations will provide greater insight into potentially modifiable risk factors for osteoporosis. It is important for the prevention and treatment of osteoporosis.

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