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The importance of 8993C>T (Thr399Ile) TLR4 polymorphism in etiology of osteoporosis in postmenopausal women

Znaczenie polimorfizmu 8993C>T (Thr399Ile) TLR4 w etiologii osteoporozy u kobiet po menopauzie

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Abstract

Introduction: *Toll-like receptors (TLR) may play a key role in initiating cellular signaling pathways by increasing the levels of inflammatory cytokines which, cooperating with osteoclasts, influence bone turnover. Numerous research articles focused on the genetic background of this condition, among others on polymorphic variants in TLR genes. The aim of the study was to examine the role of 20877G>A (Arg753Gln) in TLR2 gene and 8993C>T (Thr399Ile) in TLR4 gene in the etiopathogenesis of postmenopausal osteoporosis in Polish women.*

Material and methods: *This study included 180 postmenopausal women (t-score ⩽ -2.5), 153 postmenopausal women with osteopenia (t-score between -2.5 and -1), and 91 postmenopausal healthy women with correct t-score (t-score >-1). The 20877G>A TLR2 and 8993C>T TLR4 polymorphisms were determined by PCR/RFLP analysis.*

Results: *The analysis did not reveal statistically significant differences in the distribution of genetic variants of 20877G>A TLR2 polymorphism between the investigated groups of women. The most interesting results were connected with 8993C>T TLR4 polymorphism. Comparison of the group with osteoporosis and controls revealed overrepresentation of heterozygous 8993CT genotype (13.3 vs. 5.5%, OR=2.65, p=0.03). Also, mutated 8993T allele was overrepresented in the group with osteoporosis (6.7 vs. 2.7%, OR=2.52, p=0.04). Higher frequency of heterozygous 8993CT genotype (13.3 vs. 4.6%, OR=3.21, p=0.004) and mutated 8993T allele (6.7 vs. 2.3%, OR=3.05, p=0.005) was noted in osteoporotic women as compared to the group with osteopenia. Higher frequency of heterozygous 8993CT genotype (13.3% vs. 5.3%, OR=2.73, p=0.003) and mutated 8993T allele (6.7 vs. 2.7%, OR=2.61, p=0.004) was observed in the group with osteoporosis as compared to women with osteopenia and with correct t-score.*

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Conclusions: *Results of our study suggest an important role of mutated 8993T allele of 8993C>T TLR4 polymorphisms in the etiology of postmenopausal osteoporosis. Nevertheless, this observation requires further investigation with larger sample size comprised of Polish women.*

Key words: **osteoporosis** / **Toll-like receptors** / **genetic polymorphism** /

Streszczenie

Wstęp: *Receptory Toll-like (TLR - Toll-like receptors) mogą odgrywać kluczową rolę w inicjowaniu ścieżki sygnalizacji komórkowej, zwiększając poziom cytokin zapalnych, które współdziałając z osteoklastami modulują obrót kostny. Wiele badań koncentruje się na genetycznych podstawach tego zagadnienia między innymi wskazując udział polimorfizmu genów receptorów Toll-like. Celem pracy było zbadanie roli polimorfizmów 20877G>A (Arg753Gln) w genie TLR2 oraz 8993C>T (Thr399Ile) w genie TLR4 w etiopatogenezie osteoporozy w okresie po menopauzie w grupie kobiet polskich.*

Materiał i metody: *Badaniami objęto 180 kobiet po menopauzie z osteoporozą (t-score ⩽ -2,5), 153 kobiet po menopauzie z osteopenią (t-score pomiędzy wartościami -2,5 a -1) oraz 91 zdrowych kobiet po menopauzie z prawidłową wartością t-score (t-score >-1). Polimorfizmy 20877G>A TLR2 oraz 8993C>T TLR4 były analizowane z zastosowaniem metody PCR/RFLP.*

Wyniki: *Analiza nie wykazała statystycznie istotnych różnic w rozkładzie wariantów genetycznych polimorfizmu 20877G>A TLR2 pomiędzy badanymi grupami kobiet. Najciekawsze wyniki były związane z polimorfizmem 8993C>T TLR4. Porównując grupę kobiet z osteoporozą do grupy kobiet z prawidłowym t-score, zaobserwowano statystycznie istotną przewagę heterozygot 8993CT (13,3 vs. 5,5%, OR=2,65, p=0,03). Również zmutowany allel 8993T występował z większą częstością w grupie kobiet z osteoporozą (6,7 vs. 2,7%, OR=2,52, p=0,04).*

W grupie kobiet z osteoporozą stwierdzono również wyższą częstość występowania heterozygot 8993CT (13,3 vs. 4,6%, OR=3,21, p=0,004) oraz zmutowanego allela 8993T (6,7 vs. 2,3%, OR=3,05, p=0,005) w porównaniu do grupy kobiet z osteopenią. Wykazano również wyższą częstość występowania heterozygot 8993CT (13,3 vs. 5,3%, OR=2,73, p=0,003) oraz zmutowanego allela 8993T (6,7 vs. 2,7%, OR=2,61, p=0,004) w grupie kobiet z osteoporozą w porównaniu do grupy kobiet z osteopenią i prawidłową wartością t-score.

Wnioski: *Wyniki badania wskazują na istotną rolę zmutowanego allela 8993T polimorfizmu 8993C>T TLR4 w etiologii osteoporozy po menopauzie. Obserwacja ta wymaga jednak dalszych badań w większej liczebnie grupie populacji kobiet polskich.*

Słowa kluczowe: **osteoporoza** / **receptory Toll-podobne** / **polimorfizm genetyczny** /

Introduction

Attempts to understand the role of Toll-like receptors (TLR), playing an essential role in inherited immunological response, have been among the more important scientific investigations undertaken in recent years [1,2,3,4]. TLR receptors recognize structural elements of many microorganisms. They also play an essential role in anti-inflammatory response of non-infectious origin because they identify endogenic ligands released from damaged cells (β-defensins and oxidized lipids). Also, they can probably be activated by protein molecules modified by oxidation or nitration [5,6,7]. At present, characteristics of 15 types of TLR-like receptors in humans and 12 types in mice are known [5].

TLR4 receptor, expressed in cell membrane and endolysosomes, is the first detected and the best-known human Toll-like receptor recognizing the lipopolysaccharide (LPS) of Gram-negative bacteria present in many cells of an organism, such as immunological cells and the surface of endothelium and epithelium. TLR4 expression on the multinuclear cells and monocytes is increased under the influence of such factors as: proinflammatory cytokines, i.e. interleukin 1β, tumor necrosis factor alpha and LPS, and diminished under the influence of interleukin 10 [8]. It is proposed that some polymorphisms of *TLR4* gene could be the reason of decreased cytokine answer and, consequently, higher susceptibility to infections. Moreover, it was established that TLR4 receptor plays a role in the pathogenesis of chronic inflammation, including rheumatoid arthritis, bone mineral density (BMD), and osteoporosis [1,9].

TLR2 receptors, expressed in cell membranes, recognize atypical LPS ligands and, probably, other components known as pathogen-associated molecular patterns (PAMP). Also, it was indicated that TLR2 receptors together with TLR4 enhance the synthesis of chemokines, TNF-α, IL-10 and IL-12, and influence nuclear factor NF-kB activity [10]. A key role of TLR2 receptors in diseases related to bacterial infections, such as periodontal disease, had been demonstrated as well. Although the exact mechanism connected with pathogen-induced bone loss processes

remains to be elucidated, significant amount of molecular evidence indicated that TLR2 receptors are also involved in rheumatoid arthritis and osteomyelitis [11]. Furthermore, a modification of TLR2 dependency in the acute phase of arthritis to TLR4 dependency in the chronic phase of the disease has been confirmed in rheumatoid arthritis [12].

Estrogen deficiency and impaired balance between osteoblasts and osteoclasts activity are believed to be the main cause of postmenopausal osteoporosis [13, 14]. Genetic susceptibility to osteoporosis and bone fractures have been reported by numerous studies [15,16,17,18], underlying the molecular background of osteoporosis in the association with cytokines, their receptors, bone turnover, and intracellular signaling pathways [17]. Thus, TLR receptors could play a key role in initiating cellular signaling pathways [18]. The *Thr399Ile* polymorphisms in *TLR4* gene are the most promising and fully investigated among many genetic variants described so far. Additionally, this missense mutation affects the extracellular domain of the TLR4 receptor protein and is connected with lower levels of inflammatory cytokines, lower concentrations of adhesion molecules and acute-phase factors. This could be the reason behind persistent expression of cytokines and persistent inflammation, which could lead to differentiation of macrophages into osteoclasts for a prolonged period of time and induction of osteoporotic changes [19].

The aim of our study was to examine the role of two polymorphisms: *20877G>A (Arg753Gln)* in *TLR2* gene and *8993C>T (Thr399Ile)* in *TLR4* gene in the etiopathogenesis of postmenopausal osteoporosis in Polish women.

Material and methods

The study included 180 postmenopausal women with osteoporosis (mean age 58.8±6.2 years, *t*-score ≤ -2.5), 153 postmenopausal women with osteopenia (mean age 56.4±6.7 years, *t*-score between -2.5 and -1), and 91 healthy postmenopausal women with correct *t*-score (mean age 57.6±5.6 years, *t*-score >-1). All study participants were Caucasian and of Polish origin. The subjects were recruited in The Densitometry Policlinic of the University Clinical Hospital no 3 in Poznan. All women given their written informed consent. The Bioethics Committee of the Poznan University of Medical Sciences approved of the study. The patients were recruited for the study during their control visit for routine bone mineral density examination.

The subjects underwent physical examination and medical history review. All investigated women were at least one year after the last menstruation. BMD value in the lumbar region of the spine L2-L4, *t*-score rate, as well as laboratory parameters, were evaluated in all patients. Women who had undergone ovariectomy, with hepatic or renal diseases, diabetes mellitus or other endocrine diseases were excluded from the study. None of the subjects had received any medication known to affect bone metabolism, i.e. glucocorticoids, thyroxin, anticonvulsants, bisphosphonates, calcitonin, or hormone replacement therapy. BMD value was measured by the dual energy X-ray absorptiometry (DXA) method (Lunar DPX 100, Lunar Corp., Madison, USA). Normative values were used to determine the *t*-score (as compared to young, gender-matched, normal reference Polish population).

Two polymorphisms: *20877G>A* (*Arg753Glu*) in *TLR2* gene and *8993C>T* (*Thr399Ile*) in *TLR4* gene were determined by polymerase chain reaction/restriction fragment length polymorphism (PCR/RFLP) analysis. The primers for PCR reaction, conditions of the PCR reaction for examined polymorphisms and obtained fragments after hydrolysis by restriction process were used as previously described [20]. Products of the electrophoresis were evaluated using the system of documentation and computer analysis of the UVI image (KS 4000/Image PC, Syngen Biotech Molecular Biology Instruments, USA).

SPSS 17.0 PL program was applied for statistical analysis. Statistically significant *p* value was established at <0.05. Comparison of mean values of clinical and laboratory parameters in connection with genotypes was performed by the one-way ANOVA test.

Results

As shown in Table I, there were no statistically significant differences in the distribution of genotypes and alleles of *20877G>A TLR2* polymorphism between women with and without osteoporosis. In the group with osteoporosis, the frequency of homozygous *20877GG* genotype and of heterozygous *20877GA* genotype were 92.8% and 7.2%, respectively. A similar frequency was observed in the group of women with osteopenia (*20877GG*: 93.5% and *20877GA*:-6.5%) and in the group of healthy women with correct t-score (*20877GG*: 93.4% and *20877GA*: 6.6%). In the group without osteoporosis (osteopenia and women with correct *t*-score), the prevalence of both genotypes was 93.4% and 6.6%, respectively. The same observation was connected with the frequencies of alleles of the investigated *20877G>A TLR2* polymorphism in women with correct t-score (Table I).

The most interesting results were connected with the *8993C>T TLR4* polymorphism. Comparison between the osteoporotic group and women with correct *t*-score revealed an overrepresentation of heterozygous *8993CT* genotype (13.3 *vs.* 5.5%, OR=2.65, CI 0.94-9.17, *p*=0.03). Also, mutated *8993T* allele was overrepresented in the group with osteoporosis (6.7 *vs.* 2.7%, OR=2.52, CI 0.92-8.62, *p*=0.04). Statistically significant differences were also noted between women with osteoporosis and with osteopenia. A higher frequency of heterozygous *8993CT* genotype (13.3 *vs.* 4.6%, OR=3.21, CI 1.29-9.06, *p*=0.004) and mutated *8993T* allele (6.7 *vs.* 2.3%, OR=3.05, CI 1.25-8.49, *p*=0.005) in osteoporotic women as compared to the osteopenia group was observed. Remarkable results also were obtained after comparing women with and without osteoporosis. In the group with osteoporosis, a higher frequency of heterozygous *8993CT* genotype (13.3% *vs.* 5.3%, OR=2.73, CI 1.29-6.02, *p*=0.003) and mutated *8993T* allele (6.7 *vs.* 2.7%, OR=2.61, CI 1.25-5.66, *p*=0.004) were noted as compared to women with osteopenia and with correct *t*-score (Table I).

Distributions of L2-L4 BMD values, other clinical or laboratory parameters in the investigated women in relation to particular genotypes of both, *20877G>A TLR2* and *8993C>T TLR4* polymorphisms were not significant.

Discussion

TLR2 and TLR4 are the main receptors among the TLR family. TLR receptors associated with immunological system are known to play a significant role in human body [21, 22]. Also, *TLR2* and *TLR4* genes have been revealed to take part in the aging process, formation of somatic cells and metabolic bone turnover [23, 24, 25].

Table I. Frequency of 20877G>A TLR2 and 8993C>T TLR4 polymorphisms in groups of postmenopausal women with osteoporosis, osteopenia and correct t-score.

	Women with osteoporosis n=180		*Women with osteopenia n=153*		*Women with osteoporosis – osteopenia n=333*		*Women with osteopenia and correct t-score n=244*		*Women with correct t-score n=91*	
	Observed values n (%)	*Expected values (%)*	*Observed values n (%)*	*Expected values (%)*	*Observed values n (%)*	*Expected values (%)*	*Observed values n (%)*	*Expected values (%)*	*Observed values n (%)*	*Expected values (%)*
20877G>A TLR2										
Genotypes										
GG	167 (92,8)	92,9	143 (93,5)	93,5	310 (93,1)	93,1	228 (93,4)	93,5	85 (93,4)	93,5
GA	13 (7,2)	7,0	10 (6,5)	6,4	23 (6,9)	6,8	16 (6,6)	6,4	6 (6,6)	6,4
AA	0 (0,0)	0,1	0 (0,0)	0,1	0 (0,0)	0,1	0 (0,0)	0,1	0 (0,0)	0,1
Alleles										
G	347 (96,4)	-	296 (96,7)	-	643 (96,5)	-	472 (96,7)	-	176 (96,7)	-
A	13 (3,6)	-	10 (3,3)	-	23 (3,5)	-	16 (3,3)	-	6 (3,3)	-
8993C>T TLR4										
Genotypes										
CC	156 (86,7)	87,1	146 (95,5)	95,5	302 (90,7)	90,9	231 (94,7)	94,7	86 (94,5)	94,7
CT	24 (13,3)	12,5	7 (4,6)	4,4	31 (9,3)	8,9	13 (5,3)	5,2	5 (5,5)	5,2
TT	0 (0,0)	0,4	0 (0,0)	0,1	0 (0,0)	0,2	0 (0,0)	0,1	0 (0,0)	0,1
Alleles								-		
C	336 (93,3)	-	299 (97,7)	-	635 (95,3)	-	475 (97,3)	-	177 (97,3)	-
T	24 (6,7)	-	7 (2,3)	-	31 (4,7)	-	13 (2,7)		5 (2,7)	-

Osteoclasts play a pivotal role in bone remodeling through their influence on the resorption process. The first step in osteoclasts formation is a fusion of mononuclear phagocytes. In maturated osteoclasts, the activation of appropriate signaling pathway is initiated after stimulus such as from the TLR receptors and LPS ligand or IL-1 and its IL-1R receptors. This response increases the osteoclastogenic activity through activation of IL-1R-associated kinases (IRAK-4 and IRAK-1), TNF receptor-associated factor (TRAF6), mitogen-activated protein kinases (MAPKs), and leads to the activity of osteoclasts. It is believed that these processes are over-activated in the development of postmenopausal osteoporosis [26]. This complicated process is also mediated by NFkappaB activity that induces IL-1 production. That cytokine increases the differentiation process and enhances the activity of osteoclasts. Additionally, this process could be modulated by IL-1R-associated kinase (IRAK-M) which is expressed in osteoclasts. Accelerated osteoclastogenesis and augmentation of half-life of osteoclasts have been observed in the absence of IRAK-M [27]. Increased activity or number of osteoclasts lead to the development of osteoporosis. Inflammatory cytokines have been shown to cooperate in osteoclasts relations during bone turnover [28]. Considering the above mentioned facts, TLR receptors, through their regulating inflammatory function role, play an important role as regulators in the bone turnover process [29, 30]. Due to their unique features, these receptors are also an important target of possible therapeutic interventions [8, 31, 32].

TLR polymorphisms were investigated in relationship to many different conditions directly connected with osteoporotic risk. The studies by Pahwa et al., demonstrated *Asp299Gly TLR4* polymorphism to be connected with body mass index and lung function [33]. Other studies also demonstrated a correlation between *TLR2* and *TLR4* gene receptors polymorphism, BMD value, and the development, as well as progress, of postmenopausal osteoporosis [34,35,36]. Santos et al., studied the role of *Asp299Gly* and *Thr399Ile TLR4* polymorphisms in the development of osteoporosis and obesity in Chilean women. They found

no significant relationship between *TLR4* gene receptor polymorphisms and obesity, BMD value and osteoporosis in the studied population [37]. A very interesting study by Ozkan et al., showed an association between both, *Asp299Gly* and *Thr399Ile TLR4* polymorphisms and L2-L4 BMD in postmenopausal Turkish women [18].

Despite efforts, the exact relationship between the role of TLRs genetic variants and osteoporosis remains unclear. Johnson et al., connected BMD value in mice with *C3H/HeJ* mutation in *TLR4* gene causing the depletion of *TLR4* gene function with control strains. They showed that in the mutated mice the bones were stronger and with higher BMD, as well as the content of fat tissue was diminished. Mice with impaired *TLR4* gene function showed also higher resistance to bone fractures than wild strain mice in the same physical activity. Until now, such dependency in humans has not been revealed [38].

In the present study, we analyzed *TLR2* receptor gene *20877G>A* and *TLR4* receptor gene *8993C>T* polymorphisms. Our analysis did not reveal statistically significant differences in the distribution of *TLR2* receptor gene *20877G>A* genotypes and alleles between women with or without osteoporosis. With regard to the *8993C>T TLR4* polymorphisms, we observed a statistically significant prevalence of *8993CT* genotype and *8993T* allele in postmenopausal women with osteoporosis as compared to the women with BMD *t*-score of > -2.5. This fact may suggest that mutated *8993T TLR4* allele is associated with risk of developing postmenopausal osteoporosis. Regardless, lack of association between particular genotypes of both investigated *TLR2* and *TLR4* polymorphisms and BMD value was observed in our study.

Our findings allow us to hypothesize that *TLR4* polymorphism could be of importance for the etiology of osteoporosis in Polish women. However, additional studies are necessary to investigate the issue further.

Conclusions

The results of our study suggest an important role of mutated *8993T* allele of *8993C>T TLR4* polymorphisms in the etiology of postmenopausal osteoporosis. Nevertheless, our findings require further investigations with large sample size of Polish women.

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