Original Articles/นิพนธ์ต้นฉบับ

Postmenopausal Osteoporosis in Relation to Toll-like Receptor-7 and HLA DQB1 Gene Expression on Peripheral Blood Mononuclear Cells

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Abstract

Background: Data from genome wide association study have demonstrated that the toll-like receptor-7 gene (TLR7), a well-known role in innate immunity, may participate in the development of postmenopausal osteoporosis. The HLA DQB1 gene is part of human leukocyte antigen (HLA) complex. HLA DQB1 plays a critical role in the immune system.

Objective: To determine the association of postmenopausal osteoporosis and transcripts of TLR7 and HLA DQB1 from peripheral blood mononuclear cells (PBMCs).

Methods: The study groups included 25 postmenopausal women with normal bone mineral density (BMD) and the other 25 women with osteoporosis. Blood was drawn from the subjects for bone markers and PBMCs preparation. RNA was then isolated and quantitative RT-PCR was performed for TLR7 and HLA DQB1 genes.

Results: PBMCs expression of both TLR7 and HLA DQB1 were not significantly different between postmenopausal women with normal BMD and those with osteoporosis.

Conclusions: It is unlikely that TLR7 and HLA DQB1 contribute a major role on postmenopausal osteoporosis in Thai women.

Keyword: Postmenopause, osteoporosis, women, toll-like receptor-7, expression

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Introduction

Postmenopausal osteoporosis is a systemic bone disease that is determined by changes in skeletal metabolism and architecture after menopause and increased risk of fractures[1,2]. This disease has become one of the most important public health problems in our ageing society due to the medical, psychological and economic consequences[3]. Genetic and hereditary factors, hormonal status, dietary habits and lifestyle may affect the development of postmenopausal osteoporosis. Estrogen insufficiency resulting from menopause is one of the major underlying factors of bone formation and bone resorption in women. This relates to apoptosis of osteoblast and production of pro-inflammatory cytokines, interleukin, tumor necrosis factor, macrophage colony stimulating factor, and granulocyte macrophage colony stimulating factor[4-6]. Pro-inflammatory cytokines play a significant role in the osteoporosis pathogenesis related to the osteoclast activity involving bone turnover process[7,8]. Bone turnover and inflammatory cytokines, their receptors, and intracellular signaling pathways have been recently examined for their relationship with regard to osteoporosis[9].

Toll-like receptors (TLRs), a family of pattern recognition receptors (PRRs), are single cell membrane receptors present on the specific antigen presenting cells such as macrophage and dendritic cells. They contribute a significant role in innate immune response by recognizing the conserved microbe molecule and initiate cellular signaling pathways. The activation of immune response genes by TLRs, including pro-inflammatory cytokines, constitutes a primary host defense mechanism against infectious and non-infectious agents[10,11]. TLR also plays a critical role in the differentiation and activation of osteoclasts. Ten subgroups of TLRs have been identified in human. Each TLR recognizes structurally conserved molecules derived from microbes[12,13]. TLR 3, 7, 8 and 9 are confined in the endoplasmic reticulum and endosomes, or lysosomes. Interestingly, TLR 7 recognizes single strand RNA (ssRNA). In human, host detects ssRNA viruses, for example, HIV, influenza, and vesicular stomatitis virus, through TLR 7[14,15]. The most frequently examined genetic factors of osteoporosis involved the immune system, inflammatory cytokines and their receptors, which supposed to regulate the osteoblastic and osteoclastic activity. Toll-like receptors 7 (TLR-7) enhances mediation of several inflammatory cytokines such as TNF-α, IL-6, IL-12 and IFN by recognizing single-stranded RNA in endosome which is a specific feature of viral genome processed and presented by macrophages and dendritic cells[23]. TLR-7 is reported to play a role in autoimmune disease such as systematic lupus erythematous (SLE). Previous reported showed that TLR 2, 3, 4, and 9 inhibited osteoclast differentiations and prevented the pathogenic effects of microbial invasion on bone tissues[24].

Human major histocompatibility complex (MHC) molecules are located on the surface of antigen presenting cells and involved in the presentation of antigens to T lymphocytes, NK cells, and B cells[16,17]. MHC is found in immune system in all vertebrae while human leucocyte antigen (HLA) system is a human version of MHC. HLA system has been reported to be strongly associated with metabolic disorders and autoimmune diseases (AIDs) including rheumatoid arthritis, ankylosing spondylitis, type 1 diabetes, multiple sclerosis, myasthenia gravis, celiac disease, and systemic lupus erythematosus[18,19]. HLA system comprises MHC class I and II. MHC class I presents antigen inside the cell to immune cells, while MHC class II presents antigen outside the cell to T-lym-

phocytes. MHC class II is composed of HLA-DP, DM, DOA, DOB, DQ, and DR. HLA-DQB1 is a heterodimer consisting of an alpha (DQA) and a beta chain (DQB) anchored in the membrane.

Postmenopausal osteoporosis has been studied for a long time. However, its exact etiology and pathogenic mechanisms remain inconclusive. Therefore, examination of causative genes in bone metabolism is a gap of research with great potentials. Data from genome wide association study by Nimitpong et al[27] demonstrated that genetic variation, especially single nucleotide polymorphism (SNP), is associated with bone mass in postmenopausal women. This study recruited 530 postmenopausal women and high throughput single nucleotide polymorphism (SNP) was performed. Informative SNPs were geno-typed. Of all the TLR genes screened, only an A>T change at rs1634322 SNP in the TLR7 gene passed the screening criteria. In addition, the rs1634322 in the TLR7 gene was associated with BMD at both femoral neck and lumbar spine independently of age and body weight[27]. Therefore, it seems that genetic factor of the TLR7 gene plays an important role for postmenopausal osteoporosis. However, no functional study was performed to validate the genetic variation result. Therefore, the aim of this study was to analyze the association of the two genes with postmenopausal osteoporosis by determining the expression levels of TLR-7 and HLA-DQB1 on peripheral blood mononuclear cells (PBMCs) prepared from postmenopausal women with osteoporosis and normal BMD.

Materials and Methods

Specimens

We included 25 post-menopausal osteoporotic patients (OP) with T-score of bone mineral density (BMD) measurement < -2.5 SD measured by Dual-energy X-ray absorption (DEXA) and 25 healthy post-menopausal women, the control group, T-score on BMD measurement > -1.0 SD. Detailed medical history of the subjects was obtained. The subjects did not receive hormonal treatment, tibolone, and anti-osteoporotic medication such as bisphosphonate, selective estrogen receptor modulator, strontium ranelate and calcitonin. Blood was drawn from individual participants for analysis of bone turnover markers such as total procollagen type I aminoterminal propeptide (total P1NP) and beta-cross laps (beta-CTx). Participants provided their written informed consent to participate in this study. The study was approved by Ramathibodi Hospital Ethics Committee on Human Experimentation.

BMD Measurement

Dual x-ray absorptiometry (DXA) scanning is standard for assessing bone mineral density (BMD). Areal BMD (g/cm^2) at L2-L4 of the lumbar spine femur neck and total hip was measured by dual energy X-ray absorptiometry (DEXA). Densitometers were calibrated daily.

RNA isolation and quantitative PCR

RNA was extracted from peripheral blood mononuclear cells (PBMCs). PBMCs separation from whole blood was accomplished through density gradient centrifugation using Ficoll by standard procedures. Total RNA was extracted from PBMCs using the RNeasy mini kit (Qiagen, Hilden, Germany). The concentration and quality of RNA were determined. Total RNA was reversed transcribed to generate cDNA archive using ImProm-II™ Reverse Transcription System (Promega, Madison, WI). Quantitative real-time reverse transcriptase-polymerase chain reaction

Table 1 The primer used for amplification.

TLR-7	Forward primer	5' TGCCATCAAGAAAGTTGATGCTAT 3'
	Reverse primer	5' TGGAATGTAGAGGTCTGGTTGAA 3'
HLA DQB1	Forward primer	5' TAGCAACTGTCACCTTGATG 3'
	Reverse primer	5' CGAAATCCTCGGGAGAGT 3'
Beta-actin	Forward primer	5' TCCTTCCTGGGCATGGAG 3'
	Reverse primer	5' GATGTCCACGTCACACTTCA 3'
GAPDH	Forward primer	5' TCTCTGCTCCTCCTGTTC 3'
	Reverse primer	5' ACCAAATCCGTTGACTCC 3'

(qRT-PCR) was performed by CFX 96 Real Time PCR instrument (Biorad, USA) using the SoFast^{M} EvaGreen supermix (Biorad) and primers as presented in Table 1. The PCR reactions were performed for 35 cycles at 95 °C for 3 min, 59 °C for 5 sec using primers specific for TLR-7, HLA DQB1, Beta-actin and GAPDH. Melting curve analysis of the PCR amplicons enabled identification of individual gene.

Statistical analysis

The results were analyzed using descriptive statistics for basic demographic clinical and surgical characteristics. Statistical analysis was performed using SPSS 18.0 (SPSS, Inc., Chicago, Illinois, USA). The geometric means of gene expression relative to the endogenous genes were calculated. Results were expressed as mean and standard deviation or number and percentage whatever appropriate. Differences between means were analyzed by Mann-Whitney U-test according to the distribution of data. *P* values < 0.05 were considered significant for all analyses.

Results

The demographics and clinical characteristics of the samples are presented in Table 2. For both groups, the mean menopausal period was at least 2 years. Mean age (SD) of 25 postmenopause osteoporosis and 25 non-osteoporotic patients were 60.24 (3.39) years, and 57.84 (7.62) years, respectively. There were no significant differences among groups for age, body weight and body mass index (Table 2). T-score of osteoporotic patients were -1.5, -1.8 and -0.7 at the lumbar spine, femur neck and total hip, respectively. The non-osteoporotic group consisted of women whose T score were higher than -1. The comparison of BMD was significantly different between the osteoporosis and non-osteoporotic groups (Table 3).

The results of serum bone biochemical markers, total P1NP and β-cross laps, for osteoporotic and non-osteoporotic groups are shown in Table 4. The total P1NP of postmenopause osteoporotic and non-osteoporotic groups were 53.53±17.76 and 49.56±20.61 ng/ml. The serum β-cross laps of postmenopausal osteoporotic and non-osteoporotic groups were 0.51±0.18 and 0.38±0.16 pg/ml. The results of PBMCs expression of both TLR7 and HLA DQB1 were not significantly different between postmenopausal women with normal BMD and those with osteoporosis (*P*=0.567 and *P*=0.248, respectively, Fig 1).

Table 2 Demographic and characteristics of all samples in the study

Characteristics*	NOP (n = 25)	OP (n = 25)	*P value*
Age (years)	57.84±7.62	60.24±3.39	0.09
Menopause status			
Natural (n)	21	20	0.71
Surgical (n)	4	5	
Menopause Age (years)	49.52±3.72	50.36±4.88	0.52
Body weight (kg)	65.01±9.12	52.74±5.47	0
BMI[a] (kg/m^2)	26.26±3.31	22.56±2.6	0

NOP, non-osteoporotic group; OP, osteoporotic group; BMI, body mass index; kg, kilogram; m, metre

* Values are expressed as mean ± SD

Table 3 Comparison of bone mineral density parameters

Characteristics*	NOP (n = 25)	OP (n = 25)	*P value*
Lumbar spine			< 0.001
T score	0.1±0.7	-1.5	
Femur neck			< 0.001
T score	0.4	-1.8	
Total hip			< 0.001
T score	0.7±0.6	-0.7	
Lumbar spine			< 0.001
BMD (g/cm^2)	1.022±0.080	0.748±0.097	
Femur neck BMDa (g/cm^2)	0.783±0.057	0.556±0.058	< 0.001
Total hip BMD[a] (g/cm^2)	0.936±0.066	0.706±0.066	< 0.001

NOP, Non-osteoporotic group; OP, osteoporotic group; BMD, body mineral density; g, gram; cm, centimetre

* Values are expressed as mean ± SD

Table 4 Comparison of bone biochemical markers between the groups

Characteristics*	NOP (n = 25)	OP (n = 25)	*P value*
Serum total P1NP (ng/mL)	49.56±20.61	53.53±17.76	0.47
Serum β-cross laps (pg/mL)	0.38±0.16	0.51±0.18	0.01

NOP, non-osteoporotic group; OP, osteoporotic group; P1NP, procollagen type 1 aminoterminal propeptide

* Values are expressed as mean ± SD

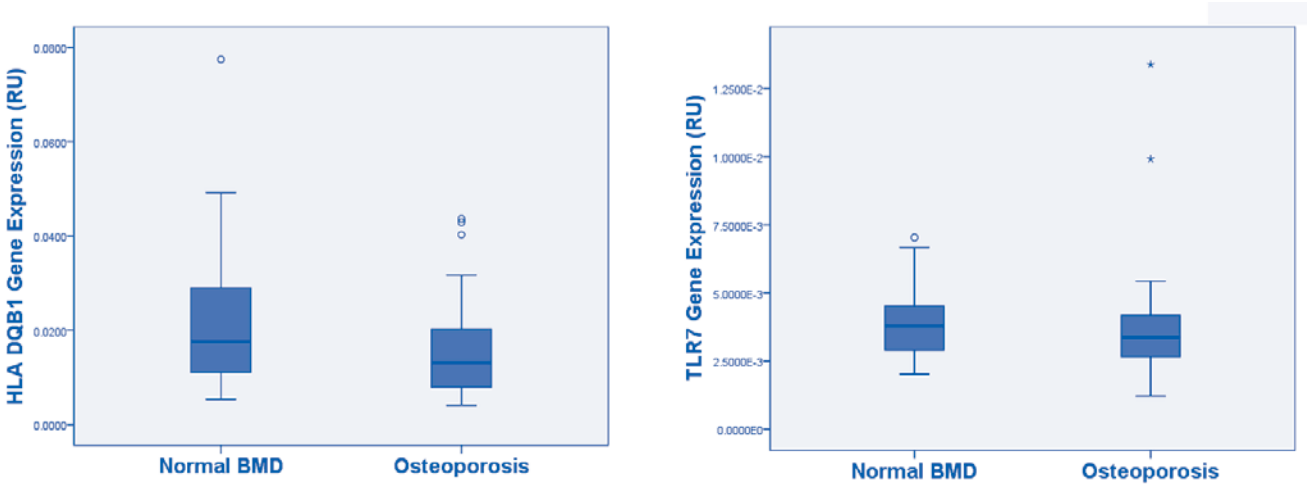

Figure 1 *TLR-7 and HLA DQB1 gene expression on PBMCs between postmenopausal women with normal BMD and the ones with osteoporosis.*

Discussion and Conclusion

Decrease in bone mass and loss of micro-architectural integrity are the significant characteristics of the osteoporosis, an asymptomatic systemic disease of the skeleton[20]. Postmenopausal osteoporosis is a systemic bone disease caused by multiple factors. Estrogen insufficiency is one of the major underlying factors of osteoporosis in women[3,21]. Estrogen influences on immune cells as demonstrated by the expression of estrogen receptors on monocytes, T and B lymphocytes. Moreover, estrogen deficiency is associated with increased production of pro-inflammatory cytokines[1,22].

Johnson et al showed the association of C1196T and A896G polymorphisms of the TLR4 gene with higher bone mass in an animal model[25]. Ozkan et al[26] reported that bone mineral density of osteoporosis women is significantly lower than in those of non-osteoporosis women (P=0.04). They also demonstrated the association of heterozygous genotypes of the Asp 299Gly and Thr 399Ile TLR4 polymorphisms and low bone mass[26]. The study conducted by Nimitphong et al[27] investigated the pooling DNA screening of TLR gene variants of 530 postmenopausal Thai women by microarray techniques. They found only the A/T SNP at rs1634322 in TLR7 passed the screening criteria. Individual geno-typing confirmed the difference in allele frequency between subjects whose BMD was in the lower tertile compared to the higher tertile (P<0.05). They concluded that a genetic variant in TLR7 gene was associated with low bone mass in Thai postmenopausal women[27]. In the present study, we explored the PBMCs expression of both the TLR7 and HLA DQB1 genes using RT-PCR. The results did not demonstrate any significant differences of PBMCs expression of genes between the normal BMD and those with osteoporosis. Miyamoto et al showed that R848, a ligand for toll-like receptor 7, mediates the inhibitory effects on osteoclast differentiation of mature osteoclasts of mouse BMMs and human PBMCs[28]. Currently, no study except that of Miyamoto et al examined the association between TLR7 and postmenopausal osteoporosis. There are many different aspects between the present study

and Miyamoto's study. Although they explored the effect of TLR7 inhibitory agent on human immune cells like ours, it was an *in vitro* study. The methodology of both studies was different. The present study was cross-sectional one, whereas Miyamoto's was experimental one. However, the approach of Miyamoto's is interesting for future study. There are many hypothetical explanations for the present result. Since the TLR-7 has been previously reported to be involved in autoimmune disease, the association of TLR-7 and bone mass may be found in some populations such as HIV and SLE patients. The induction of TLR-7 expression with pathogen such as viral RNA may be necessary for assessment.

The association between the HLA system and osteoporotic disorder has been reported in several studies. For instance, Douroudis et al investigated the relation of HLA alleles and postmenopausal osteoporosis. Their study showed that HLA B7 (P= 0.069), -DR15 (P=0.019) and DQ6 (P=0.026) were associated with a lower BMD and that a significant association between HLA alleles and bone mass loss in the Greek populations[29]. Li et al reported HLA-A* 02:07 allele was significantly higher in patients with postmenopausal osteoporosis than in control subject (P=0.023) and HLA-B 3501 allele (P=0.033) in Chinese Han populations[30,31]. We found that the expression level of HLA DQB1 was not significantly different between postmenopausal women with normal BMD. Altogether, the results from this study suggested that it is unlikely that expression levels of HLA DQB1 or TLR7 in PBMCsare associated with bone mineral density in Thai osteo-porotic women after menopause.

Conclusion and Future Perspectives

Our studied did not demonstrate the association between the PBMCs expression of TLR7 and HLA DQB1, and postmenopause osteoporotic condition. A further investigation on the relation of Toll-like receptor, HLA genes and postmenopausal osteoporosis with larger sample size and focuses on particular subject, i.e. SLE, autoimmune diseases and HIV infection should be further conducted.

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Original Articles/นิพนธ์ต้นฉบับ

การศึกษาการแสดงออกของ Toll-like receptor 7 และ HLA DQB1 ในเซลล์เม็ดเลือดขาวและภาวะกระดูกพรุนที่มีสาเหตุจากการหมดประจำเดือน

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บทคัดย่อ

ปูมหลัง: จากการศึกษาวิจัยความสัมพันธ์ของยีนพบว่ายีนที่ควบคุมการสร้าง TLR7 มีความสัมพันธ์กับการเกิดภาวะมวลกระดูกต่ำในสตรีที่หมดประจำเดือนแล้ว ส่วนยีน HLA DQB1 มีการรายงานพบว่ามีความเกี่ยวข้องกับโรคที่เกี่ยวข้องกับระบบภูมิคุ้มกัน

วัตถุประสงค์: เพื่อศึกษาการแสดงออกของยีน TLR7 และ HLA DQB1 จากเม็ดเลือดขาวซึ่งเปรียบเทียบระหว่างสตรีหมดประจำเดือนที่มีภาวะกระดูกพรุน และสตรีหมดประจำเดือนที่มีภาวะมวลกระดูกปกติ

วิธีการศึกษา: กลุ่มการศึกษาแบ่งเป็น 2 กลุ่ม กลุ่มละ 25 ราย คือสตรีหมดประจำเดือนที่มีภาวะกระดูกพรุน และสตรีหมดประจำเดือนที่มีภาวะมวลกระดูกปกติโดยแบ่งกลุ่มจากการวัดค่าความหนาแน่นของมวลกระดูก และทำการแยกเซลล์เม็ดเลือดขาวเพื่อสกัดอาร์เอ็นเอและนำไปใช้ศึกษาการแสดงออกของยีน TLR-7 และ HLA DQB1 โดยใช้เทคนิค Quantitative RT-PCR

ผลการศึกษา: การศึกษาการแสดงออกของยีน TLR-7 และ HLA DQB1 ไม่มีความแตกต่างกันอย่างมีนัยสำคัญระหว่างกลุ่มสตรีหมดประจำเดือนที่มีภาวะกระดูกพรุน และสตรีหมดประจำเดือนที่มีภาวะมวลกระดูกปกติ (P=0.567 และ P=0.248, ตามลำดับ)

สรุปผลการศึกษา: ยีน TLR7 และ HLA DQB1 ไม่น่าจะมีบทบาทในการควบคุมภาวะกระดูกพรุนในกลุ่มสตรีหมดประจำเดือนชาวไทย

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