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Relationships between blood leukocyte mitochondrial DNA copy number and inflammatory cytokines in knee osteoarthritis*

Dong ZHAN[1,2], Aree TANAVALEE[3], Saran TANTAVISUT[3], Srihatach NGARMUKOS[3], Steven W. EDWARDS[4], Sittisak HONSAWEK[†‡2,3]

[1]*Joint PhD Program in Biomedical Sciences and Biotechnology, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand*
[2]*Department of Biochemistry, Osteoarthritis and Musculoskeleton Research Unit, Faculty of Medicine, King Chulalongkorn Memorial Hospital, Thai Red Cross Society, Chulalongkorn University, Bangkok 10330, Thailand*
[3]*Department of Orthopaedics, Vinai Parkpian Orthopaedic Research Center, Faculty of Medicine, King Chulalongkorn Memorial Hospital, Thai Red Cross Society, Chulalongkorn University, Bangkok 10330, Thailand*
[4]*Institute of Integrative Biology, University of Liverpool, Liverpool L69 7ZB, UK*
[†]E-mail: sittisak.h@chula.ac.th

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Abstract: Osteoarthritis (OA) is a degenerative articular disorder manifested by cartilage destruction, subchondral sclerosis, osteophytes, and synovitis, resulting in chronic joint pain and physical disability in the elderly. The purpose of this study was to investigate mitochondrial DNA copy number ($mtDNA_{CN}$) and inflammatory cytokines in primary knee OA patients and healthy volunteers. A total of 204 knee OA patients and 169 age-matched healthy volunteers were recruited. Their relative blood leukocyte $mtDNA_{CN}$ was assessed by quantitative real-time polymerase chain reaction (qRT-PCR), and ten inflammatory cytokines in their plasma were detected by multiplex immunoassay. Blood leukocyte $mtDNA_{CN}$ in the OA group was significantly lower than that in the control group. Leukocyte $mtDNA_{CN}$ in the control group was negatively correlated with their age (r=−0.380, P<0.0001), whereas $mtDNA_{CN}$ in the OA group was positively correlated with their age (r=0.198, P<0.001). Plasma interleukin-4 (IL-4) and IL-6 were significantly higher in the knee OA group than in the control group. The plasma IL-6 level was positively correlated with blood leukocyte $mtDNA_{CN}$ in the OA group (r=0.547, P=0.0014). IL-5 showed as a major factor (coefficient 0.69) in the second dimension of principle components analysis (PCA)-transformed data and was significantly higher in the OA group (P<0.001) as well as negatively correlated with $mtDNA_{CN}$ (r=−0.577, P<0.001). These findings suggest that elevation of plasma IL-4 and IL-6 and a relative reduction in $mtDNA_{CN}$ might be effective biomarkers for knee OA. IL-5 is a plausible factor responsible for decreasing blood leukocyte $mtDNA_{CN}$ in knee OA patients.

Key words: Inflammatory cytokine; Blood leukocyte; Knee; Mitochondrial DNA copy number; Osteoarthritis
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1 Introduction

Osteoarthritis (OA) is a typical degenerative articular disorder resulting in chronic joint pain and physical disability in the elderly. It has been characterized by fibrillation, sclerosis, osteophyte formation, and progressive destruction of the articular cartilage (Manoy et al., 2018). Multiple risk factors have been identified in the various stages of OA development such as gender, obesity, joint injury,

‡ Corresponding author
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ORCID: Sittisak HONSAWEK, https://orcid.org/0000-0003-3852-9092

senescence, and genetic factors. However, the etiology and pathogenesis of OA remain poorly understood. A number of biochemical and genetic factors have been documented as playing critical roles in the progression of OA.

Mitochondria produce energy by synthesizing adenosine triphosphate (ATP) to drive normal cellular physiological functions. Mitochondrial DNA (mtDNA) does not contain introns or histones, and the oxidative damage level may be slightly lower in mtDNA than in nuclear DNA (Lim et al., 2005). The fundamental mechanisms underlying mitochondrial dysfunction in OA involve increased chondrocyte apoptosis, decreased chondrocyte biosynthesis, cytokine-induced chondrocyte inflammation, and cartilage matrix calcification. Mitochondrial dysfunction in OA chondrocytes could stem from somatic mutations in the mtDNA or from the direct effects of proinflammatory cytokines and reactive oxygen species (ROS) (Blanco et al., 2011). The mtDNA copy number ($mtDNA_{CN}$) decreases with age in adults over fifty, and thus mtDNA biogenesis is important to maintain an appropriate $mtDNA_{CN}$ content to slow down cellular aging (Dechsupa et al., 2017). Recent studies have demonstrated that $mtDNA_{CN}$ was associated with osteoarthritis and might play a contributory role in the pathological process of OA (Fang et al., 2014; Zhan and Honsawek, 2019).

A few inflammatory cytokines or chemokines are highly expressed in the circulation system of the elderly and maintain a low-grade systemic inflammatory condition (Hsu et al., 2009; Schaap et al., 2009; López-Otín et al., 2013). Mitochondria and mtDNA content can influence low-grade systemic inflammation by various mechanisms. Systemic inflammation can be upgraded through weakening of the mitochondrial biogenesis of macrophages (Chawla et al., 2011). Recently, leukocyte $mtDNA_{CN}$ was found to be negatively correlated with high-sensitivity C-reactive protein (hs-CRP), white blood cell count, and interleukin-6 (IL-6), and associated with oxidative stress in the elderly (Tanpaisankit et al., 2017; Wu et al., 2017). Inflammatory cytokines with high expression not only affect systemic and local conditions of OA, but also are crucial in synthesizing cartilage and regulating the extracellular matrix (Kapoor et al., 2011; Mabey et al., 2016).

Both mtDNA content and knee OA are related to low-grade inflammatory reactions and the aging progress, and therefore it is necessary to investigate $mtDNA_{CN}$ and its relationships with inflammatory cytokines. The purpose of this study was to evaluate blood leukocyte $mtDNA_{CN}$ and inflammatory cytokines in patients with knee OA compared to those in a control group, and to determine the relationships between $mtDNA_{CN}$ and inflammatory cytokines in knee OA patients.

2 Materials and methods

2.1 Study population

The study was approved by the Institutional Review Board (IRB number 565/59) on Human Research of the Faculty of Medicine, Chulalongkorn University, Thailand, and was conducted in compliance with the guidelines of the Declaration of Helsinki. All subjects gave written informed consent prior to their participation in this study.

A total of 204 patients with unilateral primary knee OA (age range 50–80 years) were recruited in the OA group. OA patients were identified by diagnostic criteria of the American College of Rheumatology. Patients who had other chronic inflammatory diseases, immunological abnormalities, prior knee trauma, or knee surgery were excluded from the study. A total of 169 healthy volunteers (age range 50–80 years) with normal knee radiographs, without symptoms, signs, or a previous history of OA participated as the control group.

The knee radiographs of controls and OA subjects were categorized using the Kellgren-Lawrence (KL) classification system (Kellgren and Lawrence, 1957). The healthy volunteers had a KL grade of 0. Every patient included in the OA group had a KL grade equal to or greater than 2: 50 patients were Grade 2, 64 patients Grade 3, and 90 patients Grade 4. All patients were hospitalized at least one day before a knee arthroscopy or total knee replacement operation. The researchers were blinded as to which group each participant had been assigned. Serial numbers were randomly selected for labeling the biological samples from participants.

2.2 Sample preparation

Whole blood samples collected into sodium heparin-coated tubes (Greiner Bio-one, Chonburi, Thailand) were centrifuged at 4000*g* for 10 min to

obtain blood leukocytes. Genomic DNA was then extracted from the leukocytes using an Illustra Blood Genomic Prep Mini Spin kit (GE Healthcare, Buckinghamshire, UK). The quality and concentration of the extracted DNA were identified using a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The genomic DNA was aliquoted and stored at −80 °C until measurement. Among all participants, plasma samples of 12 healthy volunteers and 31 knee OA subjects were available for multiplex immunoassay. All plasma specimens were stored at −80 °C until further analysis.

2.3 Measurement of blood leukocyte mtDNA$_{CN}$

The relative mtDNA$_{CN}$ was determined using quantitative real-time polymerase chain reaction (qRT-PCR) as described previously (Xing et al., 2008). DNA samples were amplified in 10-μL reactions using a Step One Plus Real Time PCR system (Applied Biosystems, Foster City, CA, USA). The primer sequences for the mitochondrial nicotinamide adenine dinucleotide (NADH) dehydrogenase 1 (*ND1*) gene were: ND1 F, 5'-CCCTAAAACCCGCCACATCT-3' and ND1 R, 5'-GAGCGATGGTGAGAGCTAAGGT-3'. The primer sequences for the nuclear human β-haemoglobin (*HGB*) gene were: HGB F, 5'-GTGCACCTGACTCCTGAGGAGA-3' and HGB R, 5'-CCTTGATACCAACCTGCCCAG-3'. Both reactions contained 5 μL qPCR Green Master Mixes (2×) (Biotech Rabbit, Germany), 2 μL DNA template (1.56 ng/mL), and 0.2 μL forward and reverse primers (10 μmol/L). The thermal cycling profile for both the *ND1* and *HGB* genes started with 95 °C incubation for 30 s for 1 cycle, followed by 35 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 50 s. All amplification specificity was regulated by melting curve analysis. Threshold cycle (C_T) values were used to calculate the qRT-PCR results. An unchanged DNA sample was used as reference which was placed in the same well position of the tray. Each sample mtDNA$_{CN}$ was estimated by the comparative method using the fold induction $2^{-\Delta\Delta C_T}$ equation (Livak and Schmittgen, 2001). The ΔC_T was determined by calculating the difference between the average C_T value of the *ND1* gene and that of the *HGB* gene. The difference between the sample ΔC_T and reference ΔC_T gave the $\Delta\Delta C_T$ value.

2.4 Measurement of plasma cytokines

The levels of ten commonly tested cytokines in plasma were examined by magnetic bead-based multiplex measurement on 96-well plates (Bio-Plex Precision Pro, Bio-Rad, Hercules, USA). These 10-type beads were coupled by ten cytokine antibodies: IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-13, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α. Briefly, 50 μL of the coupled beads were added to the experimental wells. After washing, 50 μL of standards and plasma of OA patients or healthy volunteers were transferred into each well with beads and incubated for 1 h. Next, the detecting antibodies were added and incubated for 30 min. Streptavidin-phycoerythrin (streptavidin-PE) of appropriate concentration was added to each well and incubated for 10 min. Finally, assay buffer was added to resuspend the beads. A Bio-Plex 200 array reader (Bio-Plex 200 Multiplex System, Bio-Rad, Hercules, USA) was used to measure the levels of cytokines in suspension.

2.5 Statistical analysis

All qualitative and quantitative variables were analyzed with software R Version 4.3.2 and GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA). Quantitative data are presented as mean±standard error of mean (SEM). Qualitative data are presented as frequencies. The normality of the quantitative data was identified by a Shapiro-Wilk test, and an *F*-test was used to check for homogeneity of the variance. Student's *t*-test and Welch's test were used for unpaired normal data based on the variance equality. Variables not normally distributed were analyzed by the Wilcoxon test. Qualitative data were analyzed by a Pearson χ^2 test to examine the frequency. Linear correlations of two variables were analyzed by Pearson's correlation for normal data and Spearman's correlation for non-normal data. Logistic regression analysis models were performed to determine the association between mtDNA$_{CN}$ and cytokines by taking age, gender, and body mass index (BMI) as the covariates.

To investigate the interactions of multiple cytokines with mtDNA$_{CN}$ and disease, all detectable cytokines were integrated into different dimensions/components by applying principle components analysis (PCA) to reveal the potential variables. The

number of dimensions was collected from evidence of scree plots and the Kaiser-Harris criterion for eigenvalues greater than 1. A non-rotation method was used in PCA to display data features. Interpretation of each dimension was supported by a high absolute value of eigenvalue loading coefficients. The calculation of individual dimension scores for each participant was based on detectable inflammatory cytokines. Differences between the control and OA groups in each dimension were analyzed with these scores. Subsequently, the correlations between standardized $mtDNA_{CN}$ (mean=0, standard deviation (SD)=1) and individual scores of each dimension in different groups were analyzed using a simple linear model. $P<0.05$ was considered to indicate a significant difference.

3 Results

3.1 Clinical characteristics of the participants

Demographic data of the patients included in this study are displayed in Table 1. There were no significant age differences between groups ($P=0.132$). The number of females was significantly higher in the OA group than in the control group ($P<0.001$). The mean BMI was significantly lower in the control group than in the OA group ($P<0.001$).

Table 1 Basic characteristics of healthy controls and primary knee OA group

Group	Age (year)	Female/male	BMI (kg/m^2)	KL grade		
				2	3	4
Control (n=169)	62.69±0.42	111/58 (65.68%)	25.12±0.34			
OA (n=204)	63.70±0.52	168/36 (82.35%)	27.09±0.26	50	64	90
P-value	0.132	<0.001	<0.001			

OA: osteoarthritis; BMI: body mass index; KL: Kellgren-Lawrence. Data are expressed as mean±standard error of mean (SEM), number (percentage of female), or number

3.2 Correlation of $mtDNA_{CN}$ and age in patients with knee OA

The mean blood leukocyte $mtDNA_{CN}$ of the OA group was significantly lower than that of the control group (0.50±0.03 vs. 1.07±0.04, $P<0.0001$; Fig. 1). There were no significant differences in $mtDNA_{CN}$ among various KL grades of the OA group ($P=0.3277$). However, the blood leukocyte $mtDNA_{CN}$ of OA patients with KL Grade 2, 3, or 4 was significantly lower than that of controls ($P<0.0001$; Fig. 1). In the control group, the $mtDNA_{CN}$ was negatively correlated with the age of healthy participants ($r=-0.380$; $P<0.0001$). However, the $mtDNA_{CN}$ increased with age in the OA group ($r=0.198$, $P=0.0025$). Thus, the relationships between age and $mtDNA_{CN}$ showed opposing linear correlations in the control and OA groups.

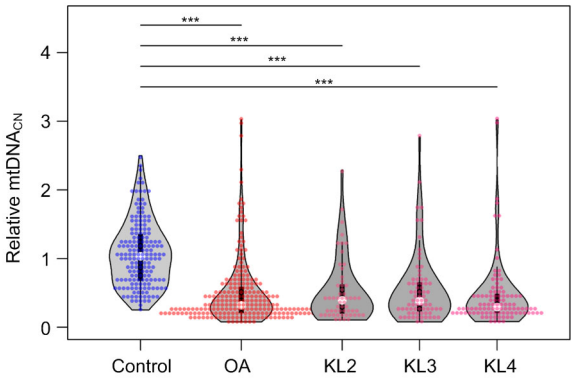

Fig. 1 Relative $mtDNA_{CN}$ of blood leukocytes in the controls and OA subjects

The relative $mtDNA_{CN}$ of blood leukocytes of the OA group was lower than that of the control group ($P<0.0001$). The blood leukocyte $mtDNA_{CN}$ of OA patients with KL Grade 2, 3, or 4 was significantly lower than that of controls ($P<0.0001$). The relative $mtDNA_{CN}$ of blood leukocytes did not differ among KL Grade 2, 3, and 4 patients in the OA group ($P>0.05$). *** $P<0.0001$ vs. control

3.3 Inflammatory cytokine levels in plasma

Plasma samples from 12 healthy volunteers and 31 OA patients were used to assess the levels of inflammatory cytokines. The levels of two cytokines, IL-4 and IL-6, were significantly greater in the OA patients than in the controls ($P=0.003$ and $P<0.001$, respectively; Table 2). The levels of IL-1β, IL-2, IL-5, IL-10, IL-12p70, IL-13, and TNF-α were higher in the OA group, but the differences were not significant. Plasma IFN-γ was undetectable in the control group, but was detected in four participants in the OA group.

3.4 Correlation of cytokines and $mtDNA_{CN}$

Since ten paired inflammatory cytokine levels and $mtDNA_{CN}$ were acquired from 43 participants, a matrix correlation analysis was applied to interpret

their mutual relationship. The correlogram of the control group is shown in Fig. 2a. Blue circles demonstrate that most inflammatory cytokines in the control group were positively correlated with other cytokines (P<0.05). In the OA group, only IL-6 was positively correlated with mtDNA$_{CN}$ (r=0.547, P=0.0014; Fig. 2b). Blank squares represent undiscovered relationships between each cytokine and mtDNA$_{CN}$. In the OA group, there were no associations between IL-6 and other inflammatory cytokines, except for IL-1β (r=0.361, P=0.0458). The other cytokines showed positive associations with each other. As age, gender, and BMI can influence the findings, we performed logistic regression analyses to determine the correlation between plasma cytokines and blood leukocyte mtDNA$_{CN}$ of OA patients by taking age, gender, and BMI as the covariates. We observed no significant correlation between blood leukocyte mtDNA$_{CN}$ and plasma cytokines in the knee OA patients.

3.5 Principle components analysis

Eigenvalues of the three dimensions were greater than 1; the first dimension had an eigenvalue

Table 2 Description of ten inflammatory cytokines and statistical analysis of comparisons between the control group and the knee OA group

Cytokine	Concentration (pg/mL)*		95% CI	P-value
	Control group (n=12)	OA group (n=31)		
IL-1β	20.550±1.666	23.354±1.724	−8.802, 3.195	0.351
IL-2	115.492±9.592	138.937±9.358	−56.257, 9.367	0.157
IL-4	2.087±0.408	4.072±0.378	−2.840, −0.530	0.003
IL-5	117.155±8.996	148.521±15.710	−49.800, 4.820	0.088
IL-6	156.740±12.953	437.826±77.224	−328.160, −73.660	<0.001
IL-10	131.324±10.341	157.557±12.856	−59.633, 7.167	0.120
IL-12p70	2.817±0.501	6.173±1.249	−4.870, 0.240	0.155
IL-13	24.658±2.025	27.539±2.032	−9.981, 4.217	0.417
IFN-γ	ND	5.385±1.061[a]	NA	NA
TNF-α	8.578±0.788	9.576±0.759	−9.981, 4.217	0.454

CI: confidence interval; ND: not detectable; NA: not available. * Data are expressed as mean±standard error of mean (SEM). [a] IFN-γ was detectable in only four knee osteoarthritis (OA) patients

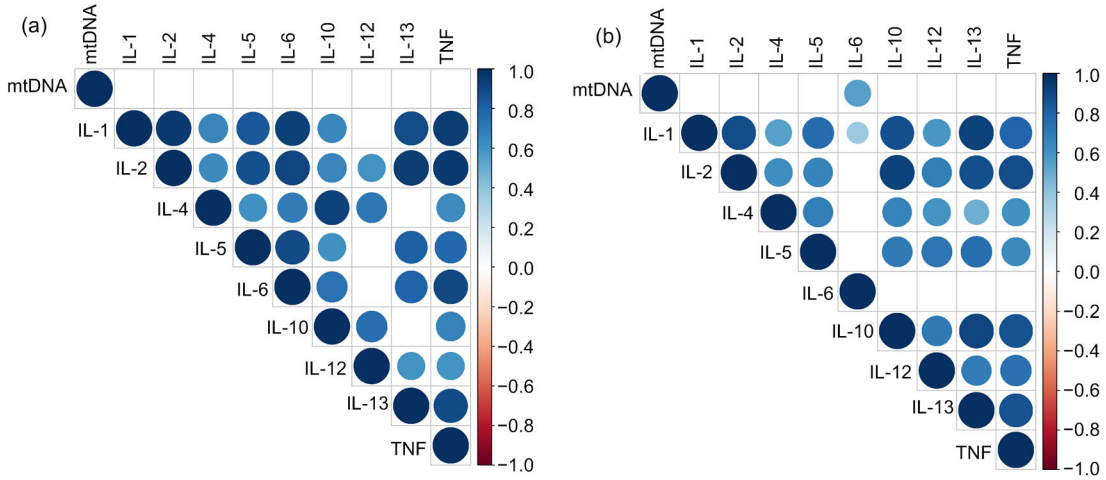

Fig. 2 Correlational matrix of mtDNA$_{CN}$ and nine inflammatory cytokines (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-13, and TNF-α)

(a) Correlogram of the control group; (b) Correlogram of the osteoarthritis (OA) group. The colored gradient legends represent coefficients of correlation r values from +1.0 (dark blue) to −1.0 (dark red). The presence of a circle indicates a significant correlation (P<0.05); conversely, blank squares indicate no significance (P>0.05). All coefficients were computed by Spearman's rho rank correlation for possible pairs of variables in the matrix

of 5.47, the second 1.11, and the third 1.06 (Fig. 3). These three dimensions accounted for 60.8%, 12.3%, and 11.7% of the variance for the nine cytokine variables, respectively, and all three cumulative dimensions accounted for 84.5% of the variance in the height variables (Table 3).

All nine detectable cytokines were positively correlated with the first dimension, which suggested that the nine variables worked together to produce the effects seen in OA. The first dimension was highly correlated with IL-13, TNF-α, IL-2, IL-10, and IL-1β whose correlation coefficients were more than 0.90. Therefore, this dimension was a predominant measurement for these five cytokines. Although correlation coefficients for IL-4 and IL-12p70 were more than 0.5, they were not considered to be the main variables in the first dimension. As the coefficient value was just 0.06 for IL-6, this dimension could not be regarded as a measure of IL-6. There were no statistical differences between the OA and the control groups in the first dimension. Also, there were no significant correlations with standardized $mtDNA_{CN}$ in either group (Table 3).

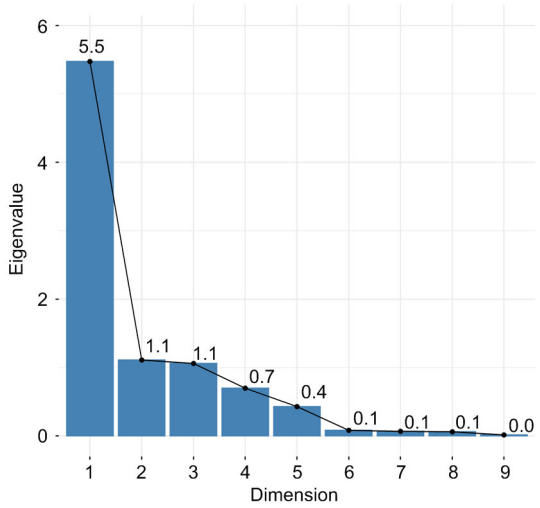

Fig. 3 Scree plot of inflammatory cytokines
The eigenvalues of dimensions 1, 2, and 3 were more than 1

In the second dimension, the coefficient for IL-5 increased to 0.69, and four cytokines (IL-5, IL-4, IL-12p70, and IL-6) were then positively correlated with this dimension (Table 3, Fig. 4a). The mean individual score for the OA group was higher than that of the control group in the second dimension. The standardized $mtDNA_{CN}$ was negatively correlated with the individual scores in the second dimension for the OA group (r=−0.577, P<0.001), but there was no significant correlation for the control group.

In the third dimension, IL-6 became the only predominant factor, with a coefficient value of 0.94. In contrast, IL-5 was negatively correlated to this dimension, with a coefficient value of 0.40 (Table 3, Fig. 4b). Other values were less than or equal to 0.11. The mean score of the control group was significantly different to that of the OA group (P<0.0001). There was no significant correlation between the cytokines studied and the standardized $mtDNA_{CN}$ for each group (Table 3).

4 Discussion

Few studies have described the mtDNA content from peripheral blood leukocytes of patients with primary knee OA. Our study demonstrated that blood leukocyte $mtDNA_{CN}$ was lower in OA patients than in

Table 3 Summary of principle components analysis in dimensions 1, 2, and 3

Dimension	Eigenvalue	Explained proportion	Scoring coefficient						
			IL-1β	IL-2	IL-4	IL-5	IL-6	IL-10	IL-12p70
1	5.47	60.8%	0.93	0.94	0.69	0.40	0.06	0.94	0.62
2	1.11	12.3%	−0.23	−0.23	0.46	0.69	0.24	−0.04	0.42
3	1.06	11.7%	0.01	0.02	0.11	−0.40	0.94	0.04	0.00

Dimension	Scoring coefficient		Comparison in 3 major dimensions			Correlation with standardized $mtDNA_{CN}$			
	IL-13	TNF-α	Control group*	OA group*	P-value	Control group		OA group	
						r	P-value	r	P-value
1	0.95	0.95	0.876±0.437	−0.339±0.454	0.1280	0.004	0.9893	−0.0860	0.6439
2	−0.23	−0.19	−0.545±0.061	0.283±0.209	0.0007	0.008	0.9936	−0.5767	0.0007
3	−0.03	−0.02	−0.453±0.029	0.175±0.210	<0.0001	−0.280	0.5272	0.1657	0.3730

IL: interleukin; TNF: tumor necrosis factor; OA: osteoarthritis. * Data are expressed as mean±standard error of mean (SEM)

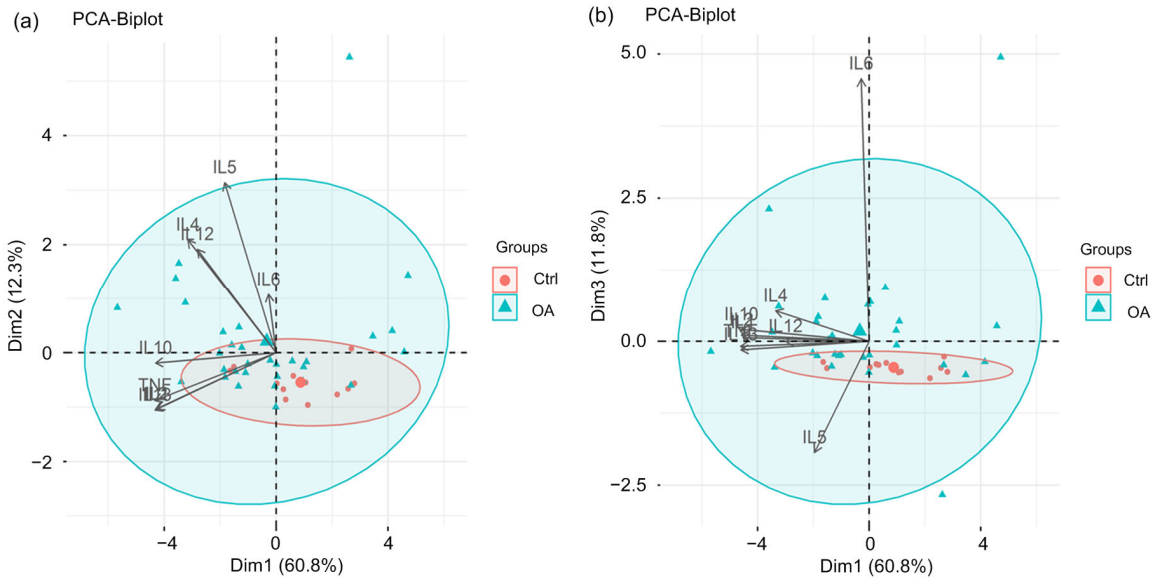

Fig. 4 Biplots showing cytokine coefficients with positive or negative effects in dimensions 1, 2, and 3

Participants' component scores were plotted against each other and in group-dependent clusters: the principle components analysis (PCA) biplot for dimensions 1 versus 2 (a) and dimensions 1 versus 3 (b). Dim: dimension; Ctrl: control; OA: osteoarthritis

healthy participants. We found that IL-4 and IL-6 concentrations were significantly higher in OA patients. Moreover, IL-6 was significantly and positively correlated with $mtDNA_{CN}$ in OA patients.

Results from the PCA indicated that underlying dimensions of multiple cytokines and $mtDNA_{CN}$ affected knee OA. Blood leukocyte $mtDNA_{CN}$ was higher in knee OA patients and inversely correlated to dimension 2 in which IL-5 was the major factor. IL-5 was higher in patients with knee OA. In dimension 3, IL-6 was the major factor and IL-6 levels were higher in the OA group. These results suggest that these cytokines may contribute to the biological characteristics of primary OA and may cause chronic low-grade inflammation.

Abnormal $mtDNA_{CN}$ can disrupt the gene expression, differentiation, and migration of normal cells (Liu et al., 2015). Mitochondria dysfunction of peripheral leucocytes may be associated with OA development by increasing ROS and apoptosis (Meyer et al., 2013; Yang et al., 2013). Previous studies found that leukocyte mtDNA was lower in patients with various diseases including metabolic syndrome, cancer, and neurodegenerative diseases (Chomyn and Attardi, 2003). Fang et al. (2014) reported that $mtDNA_{CN}$ was higher in knee OA subjects compared to controls, but the $mtDNA_{CN}$ did not differ among various subgroups of OA subjects. The explanations for these conflicting results remain unclear, but might be due to differences in clinical settings, disease advancement, populations, ethnic groups, or the assays applied. Age is an influential factor for $mtDNA_{CN}$. In the general population, mtDNA content and age show a non-linear relationship; the mtDNA content of people over 50 years old has been shown to decrease with their age (Dechsupa et al., 2017). Our findings from the healthy volunteers also showed that age was negatively correlated with relative $mtDNA_{CN}$.

Inflammation has been recognized as a contributor to the symptoms and progression of OA, especially in cartilage and the synovium (Smith et al., 1997; Blanco et al., 2011). Cytokines and mediators of local inflammatory events involve circulating blood. Numerous studies reported that several inflammation-related cytokines were higher in OA blood (Attur et al., 2012; Berenbaum, 2013; Mabey et al., 2016). Low-grade, systemic, and chronic inflammation might be a pivotal sign or factor for initiating or developing the disorder (Blanco et al., 2018).

IL-6 is a pro-inflammatory cytokine which decreases type II collagen and increases matrix metalloproteinases (Porée et al., 2008; Kapoor et al., 2011). Higher concentrations of IL-6 have been found in

plasma and sera of OA patients (Kaneko et al., 2000; Mabey et al., 2016). In a 15-year follow-up study, researchers discovered that IL-6 was consistently upregulated in patients with radiographic knee OA (Livshits et al., 2009). In contrast, lower IL-6 levels have been found to decrease the probability of development into OA by 87.01% (Goekoop et al., 2010). IL-6 is also recognized as a myokine, which increases by up to 100-fold compared to the baseline level when skeletal muscle contracts during aerobic exercise. IL-6 may be acutely secreted and have an anti-inflammatory effect (Pedersen and Febbraio, 2008). Prolonged higher levels of IL-6 in the blood can maintain low-grade, chronic, and systemic inflammation in OA patients instead of the benefits of acute IL-6 production by myocytes.

As an anti-inflammatory cytokine, IL-4 regulates macrophage action to decrease inflammation, regulate lipid accretion, inhibit the expression of a few pro-inflammatory cytokines, and increase glucose tolerance (Chang et al., 2012). IL-4 levels were higher in plasma of the OA group in this and our previous studies (Mabey et al., 2016). However, the serum IL-4 concentration was lower in the OA model of a rat study (Guo et al., 2015). These contrasting results were found in real cases of knee OA and in an animal model of OA using an identical IL-4 epitope recognized by serum IL-4 receptor and antibodies of the immunoassay. More importantly, blood IL-4 levels vary with different stages of knee OA: the IL-4 level of early knee OA (EOA) is higher than that of advanced knee OA (AOA) (Barker et al., 2014). We speculate that low-grade systemic inflammation of OA induces IL-4 release into circulating blood in EOA. However, increased IL-4 promotes an increase in soluble IL-4 receptor (IL-4R) to maintain a balanced IL-4/IL-4R system (Silvestri et al., 2006). The advantage of IL-4 effects can be blocked by increasing the levels of soluble IL-4 receptor which down-regulates IL-4 activity and quantity in AOA.

Type 2 helper T cells and mast cells express IL-5 to induce eosinophil activation, adhesion, chemotaxis, and the release of other inflammatory cytokines and chemokines (Ashraf et al., 2015). Vangsness et al. (2011) reported that the amount of IL-5 in synovial fluid (SF) in the knee was higher in AOA patients, according to the International Cartilage Repair Society (ICRS) classification. Six cytokine genes, *IL-4*, *IL-13*, *IL-5*, *IL-9*, colony stimulating factor 2 (*CSF2*), and *IL-3*, are located close together in the 5q31–33 region of the human genome, forming a cytokine cluster (Thomas et al., 1997). Thus, variation in *IL-5* could be influenced by *IL-4* gene expression. This was confirmed by a report that IL-5 production could be down-regulated in $CD4^+$ T cells of *IL-4* gene knock-out mice (Kopf et al., 1993).

Our study applied PCA to identify patterns within complicated biological data. The $mtDNA_{CN}$ has been negatively correlated with CRP-related factors such as IL-6, fibrinogen, leukocyte count, and hs-CRP in the elderly (Wu et al., 2017). Although our research did not find any correlation between leukocyte $mtDNA_{CN}$ and plasma IL-6 level in healthy volunteers, IL-6 was positively correlated with $mtDNA_{CN}$ in elderly patients with knee OA. The impact of IL-6 on $mtDNA_{CN}$ probably was influenced by IL-5. According to dimension 3 of the PCA, the scoring coefficient of IL-5 was −0.40, whereas that of IL-6 was 0.94. When IL-5 became the largest positive coefficient (0.69) in dimension 2, it was negatively correlated with blood leukocyte $mtDNA_{CN}$ in the OA group. Yousefi et al. (2008) revealed that, after stimulation of eotaxin, lipopolysaccharide (LPS), or complement factor 5a (C5a), IL-5-pre-treated eosinophils were capable of releasing mtDNA into extracellular areas resulting in a decline in intracellular mtDNA. Moreover, this process was dependent on ROS.

The current study had several limitations that should be noted. First, the sampling of a relatively small number of subjects in one single site cannot represent the general population. Larger scale, multicenter studies should be conducted to verify our conclusions. Second, the $mtDNA_{CN}$ of all participants from the controls and OA participants has been investigated, but the limited availability of plasma inflammatory cytokine data from controls and knee OA patients posed significant challenges in the study. Another caveat is the lack of data regarding in vitro cell culture models of human chondrocytes. Further studies of $mtDNA_{CN}$ and cytokines in human primary chondrocytes obtained from healthy control and OA subjects will be useful for validating the findings of this study. Lastly, the cross-sectional design prevented determination of cause-and-effect relationships, and the potential for confounding variables needs to be taken into consideration.

In conclusion, blood leukocyte $mtDNA_{CN}$ in the knee OA subjects was significantly lower than that in the controls. Plasma IL-4 and IL-6 were significantly greater in the knee OA cases than in the controls. The plasma IL-6 level was positively correlated with blood leukocyte $mtDNA_{CN}$ in knee OA. PCA showed that IL-5 was a major factor and was significantly higher in the OA patients and negatively correlated with blood leukocyte $mtDNA_{CN}$. High levels of IL-4 and IL-6 are potential biomarkers for OA disease diagnosis and pathophysiology. IL-5 could be responsible for the decline in blood leukocyte $mtDNA_{CN}$ in primary knee OA.

Contributors

Dong ZHAN and Sittisak HONSAWEK conceived and designed the experiments, performed the experimental research, analyzed and interpreted the data, and wrote the manuscript. Aree TANAVALEE, Saran TANTAVISUT, and Srihatach NGARMUKOS collected the samples, and analyzed and interpreted the data. Steven W. EDWARDS contributed reagents, materials, and analysis tools and critically commented on the manuscript. Sittisak HONSAWEK contributed reagents, materials, and analysis tools or data, and finally edited the manuscript. All authors have read and approved the final manuscript. Therefore, all authors have full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Dong ZHAN, Aree TANAVALEE, Saran TANTAVISUT, Srihatach NGARMUKOS, Steven W. EDWARDS, and Sittisak HONSAWEK declare that they have no conflict of interest.

The study protocol conformed to the ethical standards outlined in the Declaration of Helsinki of 1975, as revised in 2008 (5) and was approved by the Ethical Committee on Human Research of the Faculty of Medicine, Chulalongkorn University, Thailand. All study subjects were fully informed of the study protocol and procedures prior to participating in the study. Written informed consent was obtained from all participants before any procedures were performed.

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中文概要

题　目：膝骨关节炎患者全血白细胞线粒体 DNA 复制数量和血浆炎性细胞因子的相关性研究

目　的：本实验研究全血白细胞线粒体 DNA 复制数量（$mtDNA_{CN}$）和血浆炎性细胞因子在膝骨关节炎患者中的变化和其相关性。

创新点：探讨了老年（50~80 岁）膝骨关节炎患者白细胞 $mtDNA_{CN}$ 和血浆炎性细胞因子水平及二者的关系。

方　法：分别收集膝骨关节炎组和对照组血液样本并对膝关节评分（Kellgren-Lawren grading）。使用实时定量聚合酶链反应（qRT-PCR）检测相对 $mtDNA_{CN}$；使用多重免疫分析（multiplex immunoassay）测定血浆中 10 种炎性细胞因子水平；应用线性相关、Logistic 回归和主成分分析（PCA）揭示骨关节炎白细胞 $mtDNA_{CN}$ 和血浆炎性细胞因子的相关性。

结　论：血浆中白介素 4（IL-4）、IL-6 和全血白细胞 $mtDNA_{CN}$ 可能是膝骨关节炎有效的生物标志物；IL-5 则对 $mtDNA_{CN}$ 减少具有潜在的影响。

关键词：炎性细胞因子；血液白细胞；膝关节；线粒体 DNA 复制数量；骨关节炎