Blood leukocyte LINE-1 hypomethylation and oxidative stress in knee osteoarthritis

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ABSTRACT

Aim: Joints inflammation is one of the most pathologic processes leading to the development of osteoarthritis (OA), possibly leading to genomic instability. LINE-1 is transposable elements, and alterations in LINE-1 methylation induced by 8-hydroxy-2′-deoxyguanosine (8-OHdG) can cause genomic instability contributing to OA development. Herein, the present study examined associations between LINE-1 methylation, 8-OHdG, and knee OA severity.

Methods: LINE-1 methylation levels were measured in 104 knee OA patients and 96 healthy controls by quantitative combined bisulfite restriction analysis. 8–OHdG was investigated by ELISA. The knee OA severity was appraised by questionnaires (VAS, WOMAC, KOOS, and lequesne index) and radiological severity based on the grading of Kellgren and Lawrence (KL) standard criteria.

Key findings: Blood leukocyte LINE-1 methylation levels were significantly lower in knee OA patients than in healthy controls. Interestingly, individuals with LINE-1 hypomethylation were significantly associated with an elevated risk of knee OA. Linear regression analysis revealed that LINE-1 methylation was independently associated with KL grading of knee OA. Furthermore, plasma 8–OHdG levels in OA cases were not significantly different from those in healthy volunteers, whereas synovial fluid 8–OHdG values were considerably higher than in paired plasma specimens of the OA subjects.

Significance: This study demonstrated that LINE-1 hypomethylation in blood leukocytes was associated with increased risk and radiographic severity of knee OA, and increased synovial fluid 8–OHdG levels were observed in knee OA patients. Collectively, LINE-1 hypomethylation and elevated 8–OHdG could emerge as biomarkers indicating the severity of knee OA and may take a possible part in the pathological process of knee OA.

1. Introduction

Osteoarthritis (OA) is a degenerative joint disease characterized by cartilage degradation, subchondral bone sclerosis, osteophyte formation, and synovial inflammation, leading to joint pain and physical disability in aging populations [1]. Despite the etiopathophysiology of OA being uncertain, it has been recognized that OA stems from a variety of inherited and environmental risk factors [2]. In recent years, epigenetic mechanisms that control gene expression without changing in the DNA sequence have been reportedly implicated in regulation the expressions of several genes known to influence the onset and development of OA [3,

4]. These previous findings prompted us to hypothesize that epigenetic alterations may be the possible pathologic events in knee OA.

DNA methylation, one of epigenetic mechanisms, is a biochemical process that transfers a methyl group to the C-5 position of the cytosine ring of DNA through the activity of DNA methyltransferases (DNMTs) [5]. Apart from its primary function in regulation of transcriptional expression, DNA methylation possesses additional effects on embryonic development, X-chromosome inactivation, and genomic imprinting [6]. Furthermore, an essential section of methylation sites throughout the human genome has been evident predominantly in transposon-derived sequences including Alu elements or short interspersed nuclear

elements (SINEs) and long interspersed nuclear elements (LINEs), which comprise roughly 11% and 17% of the human genome, respectively. Given these transposable elements accounting for more than 40% of methylation in human genome, methylation levels determined in LINE-1 have emerged as a surrogate of global genome methylation [7]. Generally, LINE-1 is an abundant and densely methylated retrotransposon across human genome. Instead, it has been reported that LINE-1 hypomethylation can cause genomic instability in several malignancies [8, 9] and autoimmune diseases [10, 11]. Interestingly, previous study showed that LINE hypomethylation was significantly correlated with its protein expression in human synovial fibroblasts of rheumatoid arthritis [12], which has been proposed as a significant mediator of synovial inflammation towards cartilage destruction in knee OA.

Although the biological relevance of changes in global DNA methylation to physiological and developmental OA remains poorly understood, oxidative stress has been shown to induce aberrant DNA methylation. In fact, oxidative stress is attributed principally to surplus generation of reactive oxygen species (ROS) that promotes a variety of DNA lesions contributing to several diseases [13]. Strikingly, it has been suggested that increased oxidative stress can induce cartilage degeneration by enhancing production of matrix metalloproteinases (MMPs) and cytokines responsible for the pathogenesis of OA [14]. A major contributor of oxidative DNA damage is 8-hydroxy-2′-deoxyguanosine (8-OHdG) that interferes with the capacity of DNA to serve as DNMT substrate, resulting in global genome hypomethylation and consequent genomic instability [15], thereby establishing an etiologic association between LINE-1 methylation and oxidative DNA damage.

Even though LINE-1 methylation levels have been studied in fibroblast-like synoviocytes obtained from patients with arthritis disease, there have been no published data regarding LINE-1 methylation in blood leukocytes of knee OA patients. Accordingly, the present study aimed to determine possible associations between blood leukocyte LINE-1 methylation, 8-OHdG, and disease severity of knee OA.

2. Materials and methods

2.1. Study participants

This study was approved by the Institutional Review Board on Human Research of the Faculty of Medicine, Chulalongkorn University (IRB 642/60). Written informed consent was obtained from all participants prior to their enrollment in the study.

A total of 104 participants diagnosed with primary knee OA in accordance with the criteria of the American College of Rheumatology and 96 healthy controls who had no symptoms or signs of OA, other arthritis, or any joint diseases were recruited in the current work. Participants having underlying diseases such as diabetes, chronic liver or renal disorders, histories of medication interfering with bone metabolism such as corticosteroids or bisphosphonates, other forms of arthritis, cancer, or other chronic inflammatory diseases were excluded from this investigation.

Knee radiography was taken when each participant was standing on both legs with fully extended knees, and the X-ray beam was centered at the concentration of the joint. The Kellgren and Lawrence (KL) classification was used to assess radiographic severity [16]. According to radiographic severity, OA patients were generally categorized into 5 KL grades (0–4): grade 0 (normal findings), no radiographic alterations; grade 1 (questionable), narrowing of the doubtful joint space and possible osteophytes around the edge; grade 2 (mild), definite osteophytes and possible narrowing of the joint space; grade 3 (moderate), multiple moderate osteophytes, definite narrowing of joint space, some subchondral sclerosis, and possible deformities in bone contour; and grade 4 (severe), marked narrowing of the joint space, large, severe sclerosis osteophytes, and deformities in bone contour. Knee OA patients enrolled in the present study were defined as having radiographic severity of KL grade ≥2 in at least 1 knee.

2.2. Clinical assessments of outcomes

The symptomatic severity of the disease - especially knee pain was evaluated by personal interview through questionnaires including visual analogue scale (VAS), Western Ontario and MacMaster University knee injury (WOMAC), knee osteoarthritis outcome score (KOOS), and lequesne index. Total VAS scores range from 0 to 10, and a higher score reflects a greater level of pain. In addition to VAS score, WOMAC can be used to determine physical limitations, and this index comprises three major categories including pain, stiffness, and physical function ranging from 0 to 100 [17]. A higher WOMAC score indicates poor outcomes including worse pain, more stiffness, and increased functional limitations. As the ability to assess the patient's opinion about their knee and associated problems, KOOS questionnaire was utilized as an instrument for determining pain severity in knee OA patients, and this questionnaire consists of five main categories including pain, other symptoms, function in daily living (ADL), function in sport and recreation (Sport/Rec), and knee related quality of life (QOL). Total KOOS scores range from 0 to 100, with a higher score indicating worse outcome [18]. Lequesne index as an index of severity for osteoarthritis for the knee can be employed to estimate the effectiveness of therapeutic interventions indicating the severity of knee pain. This index includes three subscales consisting of pain (5 items), maximum distance walked (1 item), and activities of daily living (4 items), three of which scores from 1 to 24 points based on summed responses to 10 items, with a higher score of Lequesne index indicating extremely severe knee injury [19].

2.3. DNA extraction and bisulfite modification

Whole blood samples collected into ethylenediaminetetraacetic acid-coated tubes were centrifuged at 4,000 g for 10 min to obtain blood leukocytes. Genomic DNA was then extracted from blood leukocytes using phenol/chloroform method, by which it was precipitated with absolute ethanol, glycogen, as well as 2 M sodium acetate and then incubated at -80 °C overnight. Afterwards, DNA pellets were washed with 70% ethanol, and extracted DNA was quantified by the NanoDrop-1000 (Scientific, USA). Subsequently, 100 ng extracted DNA was bisulfite converted using the EZ DNA methylation kit (Zymo Research, Orange, CA, USA), as per the manufacturer's protocol. The bisulfite-treated DNA samples were kept at -20 °C till further processing.

2.4. Quantification of LINE-1 methylation levels

LINE-1 methylation levels were quantitated by quantitative combined bisulfite restriction analysis (qCOBRA), as previously described primers and conditions [20]. Briefly, primers used to analyze LINE-1 methylation were, as follows: forward primer 5′-GTTAAAGAAAGGGGTGAYGGT-3 and reverse primer 5′-AATACRCCRTTTCTTAAACCRATCTA-3′. Each 10 μl PCR reaction contained 5 ng of bisulfite-treated DNA, 10X PCR buffer, 25 mM MgCl₂, 200 mM dNTPs, 20 μM primers, and 0.5 U HotStar *Taq* DNA polymerase (Qiagen, Valencia, CA, USA). The PCR cycling condition was performed, as follows: initial denaturation at 95 °C for 5 min; followed by 35 cycles of 95 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 45 sec; and a final extension at 72 °C for 7 min. After PCR amplification, LINE-1 amplicons (92 bp) were subsequently digested with 2 U *TaqI* and 8 U *TasI* restriction enzyme in NEBuffer3 (New England Biolabs, Ontario, Canada). The digestion reaction was incubated at 65 °C overnight, and followed by separation on an 8% non-denaturing polyacrylamide gel. Afterwards, gels were stained with ethidium bromide, and band intensities were measured by Molecular Imager Gel Doc using Image Lab Software (Bio-Rad, Begoniastraat, Belgium).

After enzymatic digestion of LINE-1 products, LINE-1 amplicons were divided into four patterns depending on methylation status of two CpG dinucleotides, as follows: two unmethylated CpGs at LINE-1 loci ($^{u}C^{u}C$); two methylated CpGs at LINE-1 loci ($^{m}C^{m}C$); and partial methylated CpGs at LINE-1 loci ($^{m}C^{u}C$ and $^{u}C^{m}C$). Measurement of LINE-1 methylation

levels from each pattern was determined by the percentage of methylation patterns in each group based on the intensity of COBRA-digested LINE-1 product. DNA fragments derived from enzymatic digestion of LINE-1 products were separated into five fragments of 92 bp, 60 bp, 50 bp, 42 bp, and 32 bp, which demonstrated different methylation patterns The number of CpG dinucleotides was evaluated by dividing the intensity of each band by the corresponding size of the double-stranded DNA fragment, as follows: A = 92 bp fragment intensity/92; B = 60 bp fragment intensity/56; C = 50 bp fragment intensity/48; D = 42 bp fragment intensity/40; E = 32 bp fragment intensity/28; and, F = [(D + E) − (B − C)]/2. LINE-1 methylation levels were calculated using the number of CpG dinucleotides, according to the following formulas: percentage of LINE-1 methylation levels ($\%^mC$) = 100 × (A + 2C + F)/(2A + 2B + 2C + 2F); percentage of hypermethylated loci ($\%^mC^mC$) = 100 × (C/2)/[(C/2) + A + B + F]; both of partially methylated loci percentage ($\%^uC^mC$) = 100 × F/[(C/2) + A + B + F]; ($\%^mC^uC$) = 100 × A/[(C/2) + A + B + F]; and, percentage of hypomethylated loci ($\%^uC^uC$) = 100 × B/[(C/2) + A + B + F]. DNA from HeLa cell line served as a positive control to standardize inter-assay variations in all measurements.

2.5. Measurement of 8–hydroxy 2′–deoxyguanosine levels

Plasma and synovial fluid 8-OHdG levels were quantified using a commercially available sandwich enzyme-linked immunosorbent assay (ELISA) kit (Trevigen, Gaithersburg, MD, USA), based on manufacturer's protocol. Antibodies specific to 8-OHdG produced by the entire immunogen were applied. Two-fold serial dilutions of 8-OHdG standard with a concentration of 0.89–56.7 ng/mL were prepared as standards. Next, the samples were measured the absorbance at 450 nm. A standard optical density-concentration curve was generated for assessment of 8-OHdG value in specimens. Intra-assay and inter-assay precision were lower than 10% and 15%, respectively. The sensitivity of this assay was 0.57 ng/ml. Afterwards, the samples were measured the absorbance at 450 nm.

2.6. Statistical analysis

All statistical analyses were performed using SPSS version 22.0 (SPSS, Inc., Chicago, IL, USA). Demographic data were compared between knee OA patients and controls using Chi-square tests and unpaired Student's *t*-tests where appropriate, and one-way analysis of variance was employed for comparisons of continuous variables among OA subgroups. Unconditional logistic regression was used to estimate associations between LINE-1 methylation and OA risk using odds ratio (OR) and 95% confidence interval (CI), with adjustments for confounding factors. Linear regression was utilized to determine possible predictors of LINE-1 methylation values as continuous variables. Pearson's correlation coefficient (*r*) was employed to determine correlation between LINE-1 methylation and 8-OHdG. Data were represented as mean ± standard error, with *P*-values less than 0.05 considered as statistically significant.

3. Results

3.1. Characteristics of study participants

The demographic and clinical characteristics of knee OA patients and healthy controls are shown in Table 1. This case-control study consists of 104 knee OA patients (83 females and 21 males, average age 70.11 ± 7.78 years) and 96 healthy controls (48 females and 48 males, average age 70.64 ± 0.63 years). No significant differences in the ages or sex ratios between knee OA subjects and healthy volunteers were observed. According to radiographic severity of knee OA, we did not find significant differences of knee pain scores corrected from numerous questionnaires including VAS, WOMAC, KOOS, and Lequesne index among knee OA subgroups (Table 1).

Table 1
Clinical characteristics and LINE-1 methylation levels in knee OA patients.

Variables	Knee OA patients				*P*-value
	Overall	KL grade 2	KL grade 3	KL grade 4	
Number	104	33	33	38	-
VAS (0–10 cm)	5.28 ± 2.42	5.13 ± 4.09	5.66 ± 0.42	5.13 ± 0.57	0.69
WOMAC					
Pain (0–4)	6.91 ± 3.26	6.55 ± 0.51	6.86 ± 0.63	7.42 ± 0.77	0.60
Stiffness (0–4)	3.58 ± 1.81	3.48 ± 0.29	3.27 ± 0.39	3.96 ± 0.39	0.40
Physical disability (0–4)	32.25 ± 15.37	29.76 ± 2.38	30.95 ± 3.50	36.50 ± 3.18	0.22
KOOS scores					
Pain	68.10 ± 15.41	70.51 ± 2.20	63.09 ± 3.45	69.27 ± 3.59	0.21
Symptom	62.51 ± 14.49	64.12 ± 2.25	63.82 ± 2.59	59.35 ± 3.51	0.41
ADL	53.16 ± 22.91	56.21 ± 3.51	54.55 ± 5.15	48.12 ± 4.93	0.39
Sport/Rec	6.23 ± 9.76	7.27 ± 1.76	4.55 ± 1.54	6.35 ± 2.21	0.60
QOL	21.02 ± 15.80	22.21 ± 2.59	24.32 ± 4.22	16.73 ± 2.45	0.22
Lesquesne index					
Pain	4.85 ± 1.67	4.79 ± 0.30	4.95 ± 0.34	4.85 ± 0.34	0.94
Maximum distance walked	3.95 ± 1.79	3.79 ± 0.32	3.91 ± 0.39	4.19 ± 0.34	0.69
Activities of daily living	5.00 ± 0.63	4.94 ± 0.86	5.14 ± 0.19	4.96 ± 0.10	0.50
Total Lesquesne index	13.80 ± 3.04	13.52 ± 0.54	14.00 ± 0.68	14.00 ± 0.57	0.78
LINE-1 methylation (%)	85.61 ± 0.48	88.74 ± 2.44	85.96 ± 1.56	81.36 ± 4.41	<0.001

*Correlation is considered statistically significant at *P*-value less than 0.05 (two-tailed).

Abbreviations: OA, Osteoarthritis; KL, Kellgren–Lawrence; VAS, Visual analogue scale; WOMAC, Western Ontario and MacMaster University knee injury; KOOS, knee osteoarthritis outcome score; LINE-1, Long interspersed nuclear element-1.

3.2. Blood leukocyte LINE-1 methylation levels of knee OA subjects and healthy volunteers

We measured blood leukocyte LINE-1 methylation levels of knee OA subjects and healthy age-matched volunteers. LINE-1 methylation values in blood leukocytes were shown to be significantly decreased in knee OA subjects compared to healthy controls (*P* = 0.02) (Fig. 1).
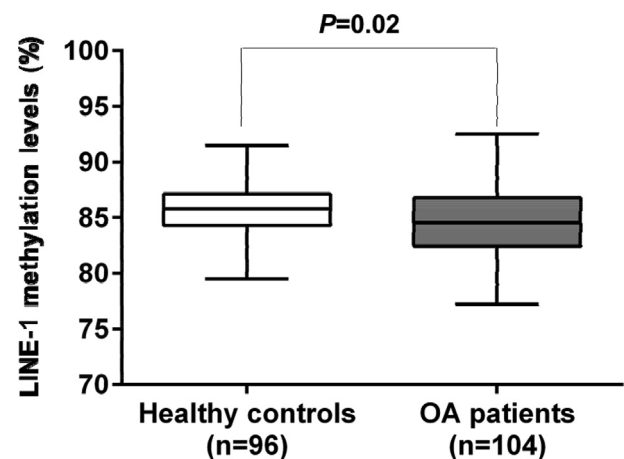

Fig. 1. Blood leukocyte LINE-1 methylation levels in healthy controls and knee OA patients.

Subsequently, we classified knee OA patients into 3 groups including the patients having radiographic severity of KL grade 2, 3, or 4. As shown in Fig. 2A, LINE-1 methylation levels in blood leukocytes of knee OA patients with KL grade 4 were significantly lower than those in patients with KL grade 2 and 3 ($P < 0.001$ and $P = 0.018$, respectively). Subsequent analysis revealed that LINE-1 methylation levels in blood leukocytes of knee OA patients were negatively correlated with the radiographic severity as shown in Fig. 2B ($r = -0.30$, $P < 0.001$).

3.3. Association between LINE-1 methylation and risk of knee OA

To investigate whether LINE-1 hypomethylayion is associated with an increased risk of knee OA, we performed an unconditional logistic regression model. After adjusting for age and gender, the analysis illustrated that there was significant association between overall LINE-1 methylation levels and knee OA risk (OR: 1.12; 95% CI: 1.00 to 1.26; $P < 0.05$), as demonstrated in Table 2. Furthermore, LINE-1 methylation levels of participants were classified into low methylation and high methylation groups by the median distribution of LINE-1 methylation in healthy controls. Interestingly, compared with individuals with high LINE-1 methylation, individuals with low LINE-1 methylation exhibited a 1.96-fold increased risk of knee OA (95% CI: 1.05 to 3.64; $P = 0.03$). Additionally, a dose-response association between LINE-1 methylation and knee OA susceptibility was estimated, in which there were significant associations between the lowest LINE-1 methylation tertile (the first tertile), the second LINE-1 methylation tertile, and an elevated risk of knee OA (OR: 2.80; 95% CI: 1.31 to 5.99; $P = 0.01$, OR: 2.17; 95% CI: 1.04 to 4.50; $P = 0.04$, respectively), when using the highest LINE-1 methylation tertile (the third tertile) as the reference group (Table 2).

3.4. Correlation between LINE-1 methylation levels and the severity of knee pain

In order to determine possible relationship between LINE-1 methylation levels and the severity of knee pain and physical disability in knee OA subjects, we conducted multiple linear regression analyses with adjustments for confounding variables. We also observed that a decrease in LINE-1 methylation levels was independently association with an increase in KL grading in knee OA patients (β-coefficient $= -0.77$, 95% CI: -4.85 to -3.03; $P < 0.001$). However, there were no significant correlations between LINE-1 methylation, age, gender, VAS, WOMAC, KOOS scores, and Lesquesne index in the patients, as depicted in Table 3.

3.5. Oxidative DNA damage in plasma and synovial fluid of knee OA patients

We further measured 8-OHdG status being a biomarker of oxidative DNA damage in plasma as well as synovial fluid of knee OA subjects ($n =$

66) and plasma of healthy controls ($n = 60$). Plasma 8-OHdG levels in knee OA patients were not significantly different with those in healthy controls ($P = 0.755$), as illustrated in Fig. 3A. On the other hand, synovial fluid 8–OHdG values were significantly higher than in paired circulating specimens of knee OA subjects ($P < 0.0001$) (Fig. 3B).

Given that oxidative stress is able to induce changes in global DNA methylation, we therefore explored an association between blood leukocyte LINE-1 methylation and 8-OHdG in knee OA subjects and also observed no significant correlation between LINE-1 methylation and circulating 8-OHdG in knee OA subjects ($r = 0.017$, $P = 0.881$). LINE-1 methylation was not correlated with synovial fluid 8-OHdG in knee OA ($r = 0.243$, $P = 0.063$).

4. Discussion

It has been recognized that inflammatory and destructive responses of the synovial membrane are the keystone events of the pathogenesis of OA, resulting in severe pain, decreased physical activity, and ultimately undergoing total joint arthroplasty [1]. Given that synovial inflammation is associated with clinical symptoms and also reflects joint degeneration in knee OA, synovium-targeted therapy could help to alleviate knee pain and perhaps to prevent structural modification in the patients. Regarding this, an increased understanding what causes synovial inflammation related to cartilage degradation in OA is of paramount importance for indicating plausible parameters and novel alternative therapies. Even though the exact mechanisms underlying synovial inflammation in knee OA are still unclear, this feature has been previously associated with genomic instability and altered expressions of specific genes known to regulate inflammatory process [21]. Epigenetic mechanisms, in particular, DNA methylation are currently known to regulate not only gene expression but also genomic stability [22], which may be implicated in OA pathology. In support of this hypothesis, main results from this study demonstrated a decline in LINE-1 methylation levels in blood leukocytes of knee OA subjects compared to healthy volunteers and its methylation levels associated with radiological severity of osteoarthritis patients. Furthermore, the present investigation measured oxidative DNA damage in the circulation and joint fluid of knee OA patients and revealed an elevation of synovial fluid 8-OHdG levels in the patients as compared with paired plasma samples. It is therefore reasonable to postulate that there exits epigenetic mechanism associated with knee OA severity – especially inflammatory process.

Given LINE-1 as transposon elements having a regulatory role in moving DNA sequences from one location on the genome to another, hypomethylation of LINE-1 elements has been shown to enhance its activity as transposon sequences, which in turn alters genomic stability and stimulates the expressions of several genes, probably implicated in a various range of pathophysiological conditions [23]. Notably, a number of studies have reported blood leukocyte LINE-1 hypomethylation in
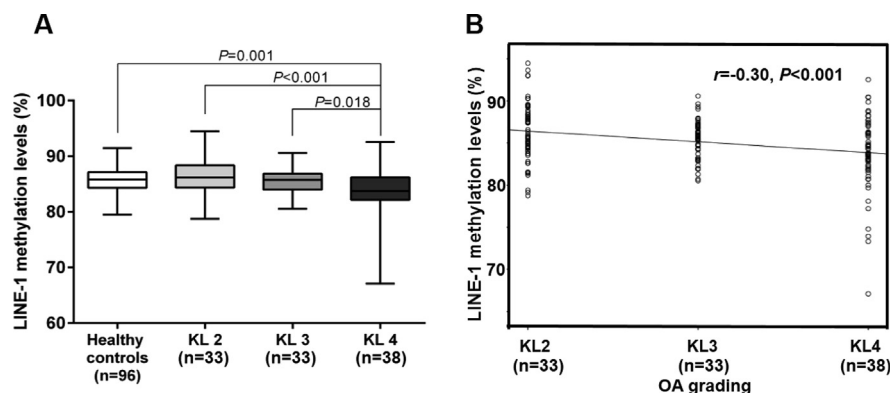

Fig. 2. Blood leukocyte LINE-1 methylation levels in subjects among different subgroups. (A) LINE-1 methylation in healthy controls and OA patients categorized by Kellgren and Lawrence (KL) classification. (B) Inverse correlation between LINE-1 methylation and disease severity.

Table 2
Association between LINE-1 methylation levels and risk of knee OA.

	Knee OA	Controls	Unadjusted OR	P-values	Adjusted OR[a]	P-value
			OR (95% CI)		OR (95% CI)	
LINE-1 elements						
Overall	100.00%	100.00%	1.150 (1.03–1.28)	0.01	1.12 (1.00–1.26)	<0.05
By median						
Low methylation	66.30%	50.00%	1.97 (1.11–3.49)	0.02	1.96 (1.05–3.64)	0.03
High methylation	33.70%	50.00%	1.00 (reference)	-	1.00 (reference)	-
By tertile						
1st tertile	56.70%	33.33%	2.95 (1.46–5.97)	0.002	2.80 (1.31–5.99)	0.01
2nd tertile	19.20%	33.33%	2.36 (1.20–4.65)	0.01	2.17 (1.04–4.50)	0.04
3rd tertile	24.00%	33.33%	1.00 (reference)	-	1.00 (reference)	-

*Correlation is considered statistically significant at *P*-value less than 0.05 (two-tailed).
Abbreviations: OA, Osteoarthritis; LINE-1, Long interspersed nuclear element-1; OR, Odd ratio.
[a] Unconditional logistic regression analysis was adjusted for age and gender.

Table 3
Multivariate linear regression analysis of blood leukocyte LINE-1 methylation levels estimates.

Variables	LINE-1 methylation levels[a]	
	ß coefficient (95% CI)	P-value
Age (years)	-0.06 (-0.14 to 0.08)	0.56
Gender	0.06 (-1.23 to 2.63)	0.47
KL grading	-0.77 (-4.85 to -3.03)	<0.001
VAS (0–10 cm)	0.11 (-0.17 to 0.56)	0.29
WOMAC		
Pain (0–4)	-1.58 (-11.86 to 7.63)	0.67
Stiffness (0–4)	-0.88 (-11.84 to 7.61)	0.67
Physical disability (0–4)	-0.09 (-0.25 to 0.20)	0.82
KOOS scores		
Pain	-0.17 (-0.12 to -0.02)	0.15
Symptom	-1.86 (-3.29 to 2.17)	0.68
ADL	-0.03 (-0.15 to 0.14)	0.93
Sport/Rec	-0.09 (-0.13 to 0.06)	0.43
QOL	0.06 (-0.04 to 0.07)	0.60
Lesquesne index		
Pain	0.02 (-0.55 to 0.66)	0.85
Maximum distance walked	0.01 (-0.52 to 0.54)	0.96
Activities of daily living	-0.02 (-1.40 to 1.16)	0.85

Abbreviations: OA, Osteoarthritis; KL, Kellgren–Lawrence; VAS, Visual analogue scale; WOMAC, Western Ontario and MacMaster University knee injury; KOOS, knee osteoarthritis outcome score; LINE-1, Long interspersed nuclear element-1.
*Correlation is considered statistically significant at *P*-value less than 0.05 (two-tailed).
[a] Unconditional logistic regression analysis was adjusted for age, gender, KL grading, and VAS.

various diseases including systemic lupus erythematosus [11], biliary atresia [20], and rheumatoid arthritis [24]. These are important evidences supporting our finding that showed LINE-1 hypomethylation in knee OA subjects. Correspondingly, LINE-1 hypomethylation was shown to be related to an increased susceptibility of knee OA. Further analysis demonstrated that LINE-1 hypomethylation was independently associated with radiographic severity of knee OA patients; however, there was no significant association between LINE-1 methylation and scores of knee pain and disability including VAS, KOOS, WOMAC, and Lesquesne index in knee OA patients. All these findings suggest that changes in LINE-1 methylation might be useful epigenetic features reflecting the severity of knee OA; nevertheless, the precise role and the diagnostic significance of LINE-1 hypomethylation in OA remain to be proven. It is notable that LINE-1 element encodes reverse transcriptase that allows it to replicate and insert itself into distinct genomic regions, which activate transcription and translation into functional proteins [25]. Interestingly, emerging evidence highlighted that alterations in DNA methylation trigger the expressions of inflammatory cytokines and matrix degradation mediators including tumor necrosis factor-alpha (TNF-α), interleukin-1beta (IL-1β), IL-6, and MMPs, which are key contributors to synovial inflammation and cartilage degradation in knee OA [26]. In view of all that has been mentioned so far, one may suppose that LINE-1 hypomethylation associated with radiographic grading in knee OA patients might be involved in stimulating the expressions of pro-inflammatory cytokines-induced synovial inflammation and catabolic genes responsible for cartilage destruction in knee OA. Collectively, our findings regarding association of LINE-1 hypomethylation with severity of knee OA provide further evidence supporting that epigenetic mechanisms, especially DNA methylation may play vital roles in OA pathogenesis.

Although the exact mechanisms behind the relevance of aberrant LINE-1 methylation to physiological and developmental OA are yet to be determined, oxidative stress has been reportedly involved in changes of
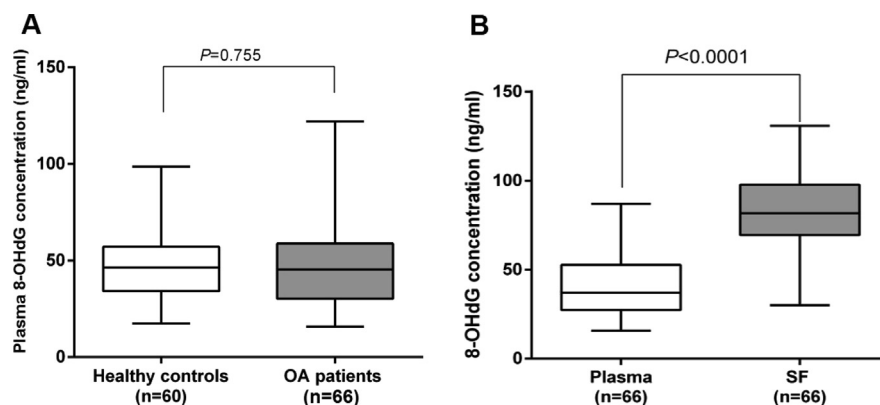

Fig. 3. 8-OHdG levels in healthy controls and knee OA patients. (A) Plasma 8-OHdG levels in knee OA patients compared with controls. (B) 8-OHdG levels in plasma and synovial fluid (SF) of knee OA patients.

DNA methylation levels [27]. It has been established that oxidative stress-induced excessive ROS production accelerates DNA reaction with the positive-charged intermediate S-adenosyl-L-methionine in DNA methylation process [28], thereby verifying the possible influence of oxidative stress in altered DNA methylation. Besides, ROS can regulate the expressions of DNMTs, which are enzymes responsible for the addition of a methyl (CH3) group to the 5th carbon atom of the cytosine residues within DNA [29]. One of the predominant forms of free radical-induced oxidative lesions is 8-OHdG that is stimulated by oxidative DNA damage resulting in inhibition of DNA methylation process at the cytosine base. In recent years, the 8-OHdG biomarker has been widely used as an indicator for the measurement of oxidative stress.

Oxidative stress can contribute to the development of various complex disorders such as musculoskeletal tumors [8, 30] and chronic liver disease [20]. Furthermore, Tanpaisankit et al. reported that neoplastic tissues in musculoskeletal tumors were associated with high tissue levels of 8-OHdG being a marker of oxidative DNA damage [30]. More recently, Dechsupa et al. found that cell senescence and 8-OHdG levels were significantly elevated in hypertrophic ligamentum flavum (LF) tissues obtained from patients with lumbar spinal stenosis lesions compared with non-hypertrophic LF tissues [31, 32]. The recent data supported our hypothesis that 8-OHdG levels in synovial fluid were locally elevated in knee OA patients. However, the previous findings are contrary to our alternative result that showed no significant difference of plasma 8-OHdG levels in knee OA group when compared to plasma control group. It seems likely that oxidative stress, particularly 8-OHdG may induce inflammatory response in the joint sites of knee OA rather than the systemic inflammation, plausibly leading to no differences in plasma 8-OHdG levels between OA patients and controls. This hypothesis is supported by our subsequent result, which noted high levels of 8-OHdG in joint fluid of knee OA as compared with paired plasma samples. Considering high levels of synovial fluid 8-OHdG in knee OA, possible explanation for this finding may result from a surplus generation of ROS-induced inflammation of the synovial membrane and further bone destruction, which leads to an increase in 8-OHdG values in joint fluid of knee osteoarthritis. However, the actual mechanisms controlling the circulating and local formation of 8-OHdG have yet to be completely elucidated. As 8-OHdG has been shown to influence alterations in DNA methylation, a possible association between aberrant LINE-1 methylation and oxidative DNA damage has been explored in this research. This study depicted no correlation of LINE-1 methylation levels with plasma and synovial fluid 8-OHdG levels in knee OA patients. In apparent contrast to this finding, our previous investigation showed an inverse association between LINE-1 methylation levels and plasma 8-OHdG levels in patients with biliary atresia [20]. The possible reason of this contrasting result may be presumably attributable to the differences in pathophysiology of the diseases between studies. Taken together, it appeared that blood leukocyte LINE-1 hypomethylation and increased levels of synovial fluid 8-OHdG might be prominent features associated with the severity of knee OA and would be of great value for their utility as biomarkers for prognosticating the severity of knee osteoarthritis.

Some inherent limitations of the current work should be acknowledged. Firstly, assessment of LINE-1 methylation levels were examined with blood leukocyte DNA, but were not measured in specific local tissues of knee OA patients due to ethical concerns about collecting synovial fluid, synovium, or cartilage specimens from participants, leading to no direct comparisons between LINE-1 methylation and 8-OHdG in those samples of controls and those of knee OA patients. Nevertheless, it has been demonstrated that LINE-1 methylation levels of blood leukocytes can reflect that of tissue-specific tumor cells in patients with musculoskeletal tumors [8]. Secondly, the total number of subjects and control participants in this work was rather limited, diminishing the statistical power and generalizability of our findings. In addition, we were unable to obtain gender-matched blood leukocytes from knee OA patients, due to high prevalence of knee OA patients in females. It is suggested that further work with more female subjects in healthy control group should

be undertaken on a prospective large-scale multi-center trial to ascertain our conclusion. Third, early onset OA patients (KL grade 0–1) were not included in the study. Therefore, we are unable to compare the LINE-1 methylation level in early onset OA patients with that in healthy subjects. Besides, incomplete assessment of potential confounders including medical comorbidities needs to be taken into account as a result of inaccessible patient records. Another caveat would be the absence of other oxidative DNA damage markers. Prospective studies should compile these measurements to additionally determine the variations between subgroups. Lastly, it should be noted that additional investigation on measurement of methylation within particular promoters or specific genes known to directly induce the pathogenesis of knee OA, especially synovial inflammation and joint destruction will help to determine aberrations in gene-specific methylation involved in OA pathology.

5. Conclusions

This is the first study to delineate that LINE-1 methylation levels in blood leukocytes were significantly decreased in knee OA patients compared to healthy controls, and its hypomethylation was markedly associated with elevated risk of knee OA. Furthermore, a reduction of LINE-1 methylation levels was observed to be independently related to the radiographic severity of knee OA. Additionally, this study reported an increase in levels of synovial fluid oxidative DNA damage in the patients when compared with paired plasma samples. Accordingly, LINE-1 hypomethylation in blood leukocytes and high levels of joint fluid 8-OHdG might severe as potential biomarkers for OA susceptibility. Additional research in possible fundamental mechanisms linked to LINE-1 methylation is warranted to fully understand the implications of epigenetic alterations in the etiopathology of osteoarthritis.

Declarations

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