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Correlation between circulating cell-free mitochondrial DNA content and severity of knee degeneration in patients with knee osteoarthritis: a cross-sectional study

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Abstract

Background Knee osteoarthritis (KOA) is characterized by mitochondrial damage and increased inflammation. Circulating cell-free mitochondrial DNA (ccf-mtDNA), which originates from damaged mitochondria, is an endogenous damage-associated molecular pattern (DAMPs) molecule that may trigger inflammation and is recognized as a potential biomarker for various diseases. In this study, we investigated the potential association between plasma ccf-mtDNA content and its use as a diagnostic biomarker in patients with KOA.

Methods We collected plasma samples from patients with KOA and healthy controls (HC). Subsequently, quantitative real-time polymerase chain reaction (qRT-PCR) was used to detect ccf-mtDNA content in the plasma samples. We used the Kellgren–Lawrence (K-L) classification criteria to classify patients with KOA into four grades: I-IV. Disease severity in patients with KOA was assessed using the Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC). Next, Spearman analysis was performed to observe the correlation between ccf-mtDNA content and the K-L classification and WOMAC score. Logistic regression analysis was used to evaluate the relationship between ccf-mtDNA and KOA risk.

Results In total, we enrolled 60 patients with KOA and HC who were matched for age, sex, and body mass index (BMI). We found that plasma ccf-mtDNA contents were significantly higher in patients with KOA (median, 2.44; quartile range, 1.10–3.79) than in HC (median, 1.08; quartile range, 0.52–2.12) ($P<0.0001$). Plasma ccf-mtDNA content sequentially increased following the KOA class I-IV group ($P=0.040$) and positively correlated with the K-L classification ($r=0.369$, $P=0.004$) and WOMAC scores ($r=0.343$, $P=0.007$). The ccf-mtDNA content did not significantly differ between patients with bilateral and those with single KOA ($P=0.083$). Patients with high levels of ccf-mtDNA had a significantly increased risk of KOA compared with those with low levels of ccf-mtDNA (odds ratio [OR], 4.15, 95% confidence interval [CI], 1.71–10.07; $P=0.002$). Quartile analysis revealed a significant dose-dependent association (P trend <0.001).

Conclusion Our study's findings showed that plasma ccf-mtDNA was highly expressed in patients with KOA compared with HC. Furthermore, ccf-mtDNA content is significantly associated with the severity and risk of KOA. Therefore, its detection may provide insight into the prevention and treatment of KOA.

Keywords Knee osteoarthritis, Circulating cell-free mitochondrial DNA, Liquid biopsy biomarker, Case–control study

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Introduction

Knee osteoarthritis (KOA) is a chronic degenerative musculoskeletal disease characterized by synovial inflammation, subchondral osteosclerosis, and osteoid formation [1]. A population-based research study showed that KOA affects 16.0% of individuals over 15 years of age, with prevalence increasing with age up to 22.9% among those aged 40 years. Furthermore, the prevalence of KOA has increased due to a developing aging population and an increase in obesity. Notably, KOA is one of the leading causes of functional impairment and chronic disability worldwide [2]. Current research suggests that the development of KOA is a complex process involving inflammatory and metabolic factors, in which chronic overload and impaired joint biomechanics lead to the destruction of articular cartilage, leading to inflammation. Subsequently, the inflammation leads to joint stiffness, swelling, and loss of mobility [3]. Early symptoms of KOA are subtle, with hidden or intermittent pain as the main symptom, whereas late KOA shows signs of deformed and enlarged joints, deformity, joint effusion, and persistent pain accompanied by joint dysfunction, such as difficulty in walking up and down the stairs and squatting. This dysfunction leads to physical disability, thus affecting the patient's quality of life [4]. KOA develops gradually; however, when obvious symptoms are seen, the disease is already in the advanced stage, with no effective treatment available to reverse the pathological changes. Therefore, early diagnosis is important for timely intervention, slowing disease progression, and improving a patient's quality of life. Currently, the diagnosis of KOA is mainly based on clinical symptoms combined with imaging. However, when imaging shows arthropathy, the disease is already in its advanced stage, causing severe and irreparable damage [5]. Consequently, there is growing interest among researchers worldwide for sensitive biomarkers that can identify disease activity and progression.

Notably, mitochondria are double-membrane organelles in the cytoplasm of eukaryotic cells that contain their DNA [6]. In addition to their primary role in energy production, they are a major source of oxidative stress from byproducts, such as reactive oxygen species (ROS) [7]. Sustained uncontrolled oxidative stress leads to increased mitochondrial membrane permeability and leakage of mitochondrial DNA (mtDNA) into the cytoplasm and extracellular space. The mtDNA is referred to as circulating cell-free mitochondrial DNA (ccf-mtDNA) and can be detected in different biological fluids (plasma or serum) [8]. Damage-associated molecular patterns (DAMPs) are molecules released in response to cellular stress or tissue injury. They are endogenous signals of danger because they induce an effective inflammatory response during non-infectious inflammation by activating the innate immune system [9, 10]. ccf-mtDNA, a DAMP originating from the mitochondria, stimulates a systemic pro-inflammatory response by activating the toll-like receptor system [11]. Therefore, ccf-mtDNA can be considered a potential liquid biopsy biomarker for mitochondrial dysfunction and oxidative stress. Moreover, liquid biopsy biomarkers based on circulating DNA samples have unique advantages over other samples because of their non-invasive nature and the ability to be repeatedly sampled [12]. Increasing evidence suggests that mitochondria play several regulatory roles in the pathogenesis of KOA, including bioenergetic metabolism, inflammatory responses, apoptosis, senescence-related responses, ROS production, and calcium metabolism [13]. Additionally, mitochondrial dysfunction and mtDNA variants contribute to cartilage degeneration and are implicated in the pathogenesis of KOA [14]. However, the role of ccf-mtDNA in KOA remains unclear.

Therefore, in the current study, we aim to investigate whether the plasma ccf-mtDNA content is potentially associated with the severity of KOA and whether it can serve as a liquid biomarker in patients with KOA.

Methods

Participants

This study was approved by the Ethics Committee of the First Affiliated Hospital of Gannan Medical University (approval number: LLSC2023-156), and informed consent was obtained from each participant. We collected samples from patients with osteoarthritis of one or both knees aged ≥ 40 years who attended the First Affiliated Hospital of Gannan Medical University between June 2023 and January 2024. Sample collection was performed following the diagnostic criteria for osteoarthritis of the knee established by the American College of Rheumatology. Participants were excluded if they had a history of knee trauma, infection, surgery, other inflammatory arthritis, autoimmune diseases, or blood-related diseases. All healthy controls (HC) were matched to the trial group for age, sex, and body mass index (BMI) and showed no evidence of clinical symptomatic changes in KOA.

The imaging severity of KOA was scored using the Kellgren (K-L) classification [15], and the severity of KOA symptoms was scored using the Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) [16]. The symptoms of KOA included knee pain, joint stiffness, and joint dysfunction. Greater pain intensity correlates with increased stiffness, and more severe joint dysfunction, resulting in higher assessment scores. For patients with bilateral KOA, only the more severely affected knee was evaluated.

Plasma sample collection

Approximately 5 mL of blood was drawn into an EDTA anticoagulant blood tube in the morning. After collection, the tube was gently inverted and mixed after collection ensuring that the anticoagulant or protective agent made full contact with the blood. Subsequently, plasma was separated from the cellular components within 2 h. Using a centrifuged pre-cooled to 4℃, the sample was first centrifuged at 1,900 g for 10 min. After centrifugation, if severe hemolysis and lipemia were observed, the sample tube was gently excluded from further analysis. The plasma supernatant was transferred to a prepared 1.5 mL enzyme-free sterilization centrifugal tube, avoiding suction to the white membrane layer during the suction process. The 1.5 mL centrifuge tube containing plasma was subjected to a second centrifugation at 16,000 g, 4 °C, for 10 min to remove cells and cell debris. Finally, the centrifuged supernatant was transferred to a new 1.5 mL enzyme-free sterilized centrifuge tube, taking care not to aspirate the precipitate, and the separated plasma was stored in a -80 °C refrigerator.

Extraction of DNA sample and quantification of ccf-mtDNA in plasma

Plasma was thawed at room temperature. Subsequently, the circulating cell-free DNA (ccf-DNA) was extracted from 1 mL of plasma using the QIAamp MinElute ccfDNA kit (Qiagen, Hilden, Germany), following the manufacturer's protocol for blood and body fluids. ccf-DNA was eluted with 55 µL of ultra-clean water. In addition, ccf-mtDNA content in the plasma was measured using quantitative real-time polymerase chain reaction (qRT-PCR) employing a modified protocol described in a previous study [17]. In this protocol, the ratio of the copy number of the mitochondrial ND1 gene to that of the human single copy of the gene 36B4 was used to determine the relative ccf-mtDNA content. In summary, the 20 µL qRT-PCR reaction for the ND1 and 36B4 genes comprised 10 µL 2 × ChamQ Universal SYBR qPCR Master Mix (Vazyme, Jiangsu, CHN), 2 µL of each primer (2 µM) (Sangong, Shanghai, CHN) and purified plasma DNA sample, and 4 µL of nuclease-free water (Solarbio, Beijing, CHN). The primer sequences are as follows: Forward primer of ND1: 5'-CCCCTAAAACCC GCCACATCT-3'; Reverse primer of ND1: 5'-GAGCGA TGGTGAGAGCTAAGGT-3'; Forward primer of 36B4: 5'-CAGCAAGTGGGAAGGTGTAATCC-3; Reverse primer of 36B4: 5'-CCCATTCTATCATCAACGGGG TACAA-3'. Additionally, the thermal cycling conditions are as follows: Pre-denaturation at 95 °C for 2 min, followed by 40 cycles, each including a denaturation step at 95 °C for 15 s, an annealing step at 60 °C for 15 s, and an extension step at 72 °C for 30 s. All samples were analyzed repeatedly using a StepOne™ qRT-PCR System (Applied Biosystems) in a 48-well plate. We used the $2^{\Delta\Delta CT}$ method to analyze relative gene expression differences. Specifically, the relative content of ccf-mtDNA in each plasma sample was calculated using the formula $2^{\Delta\Delta CT} = 2^{(CT(KOA_{ND1} - KOA_{36B4})) - (CT(HC_{ND1} - HC_{36B4}))}$, where CT represents the threshold cycle.

Statistical analysis

We used the Statistical Package for Social Sciences (SPSS) 26.0 was used to analyze the relevant data. The normality of the data was assessed using the Kolmogorov–Smirnov test and the normally distributed data was expressed as mean ± standard deviation (SD). Furthermore, comparisons between two groups were made using the student t-test, with the Student's t-test, used for unequal variance, whereas for multiple groups the one-way analysis of variance was used, with the Krukal–Wallis H-test used for unequal variance. Non-normally distributed data were expressed as medians with quartile ranges, and comparisons between the two groups were analyzed using the Mann–Whitney test. Additionally, comparisons between multiple groups were performed using the Kruskal–Wallis H test. Chi-square was used to compare qualitative data between the two groups. In addition, Spearman's correlation was used to analyze the correlation between plasma ccf-mtDNA levels and the degree of joint degeneration in patients with KOA. Notably, we used ccf-mtDNA as a categorical variable and performed the analysis using the cut-off value at the median or quartile value in the control group. Furthermore, unadjusted and adjusted odds ratios (OR) and 95% confidence intervals (CI) were determined by univariate and multivariate logistic regression analyses, respectively, to assess the correlation between plasma ccf-mtDNA levels and KOA risk. All statistical tests were conducted using a two-sided test using the significance $\alpha = 0.05$ as the significance level. $P < 0.05$ was considered statistically significant.

Results

General population characteristics

In total, we included 72 patients with KOA in this study. Of these, some patients were excluded due to missing imaging data or insufficient plasma volumes (< 1 mL). Finally, 60 patients were included, comprising 48 females and 12 males, with female composition representing 80% of the sample and an age range of 43–88 years old. Sixty HC were matched to the patients with KOA by age, sex, and BMI. Among the patients with KOA, 17 had osteoporosis, with a composition ratio of 28.33%, and 15 had hypertension, with a composition ratio of 25%. Eighteen patients had osteoarthritis of the left knee, 23 had at the

right knee, and 19 had it in both knees. Furthermore, patients with KOA were categorized following the X-ray K-L classification criteria: 9 patients had grade I, 22 had grade II, 18 had grade III, and 11 had grade IV. The mean and SD of the WOMAC total score for joint symptoms were 48.92 ± 25.62, with mean and SD of 13.78 ± 6.11, 6.43 ± 2.83, and 28.52 ± 17.21 for joint pain, joint stiffness, and functional impairment, respectively. (Table 1).

Distribution of ccf-mtDNA content in cases and controls by patient characteristics

Overall, ccf-mtDNA contents were significantly higher in patients with KOA than in HC (median, 2.44; quartile range, 1.10–3.79) vs. (median, 1.08; quartile range, 0.52–2.12) ($P < 0.0001$). In the stratified analyses, patients with KOA had significantly higher ccf-mtDNA in all strata except those with BMI > 23.69 than HC. In addition, we compared the differences in ccf-mtDNA levels between strata for each variable between patients with KOA and HC. In HC, ccf-mtDNA levels were higher in shorter subjects than in taller subjects ($P = 0.043$). The strata of other variables in the HC group did not significantly differ from all variables in the KOA group (Table 2).

Comparison of the general conditions and ccf-mtDNA content among patients with different K-L grades of KOA

Patients with KOA were divided into four groups following the X-ray K-L classification. The WOMAC scores (including pain, stiffness, dysfunction, and total score) significantly differed between the groups under the different K-L classifications ($P < 0.0001$). Furthermore, ccf-mtDNA expression increased continuously with increasing K-L grade, and the difference was statistically significant ($P = 0.040$). No statistically significant differences were observed in age, sex, height, weight, or BMI among the different K-L classifications ($P = 0.136$, 0.409, 0.261, 0.563, and 0.975, respectively) (Table 3).

Correlation analysis of plasma ccf-mtDNA contents with K-L classifications and WOMAC in patients with KOA

Results from Spearman's analysis showed that plasma ccf-mtDNA content in patients with KOA

Table 1 Characteristics of the study population

Variables	KOA ($n = 60$)	HC ($n = 60$)	*P* value
Age (mean ± SD)	60.73 ± 10.01	59.53 ± 9.54	0.503
Gender (M/F)	12/48	12/48	1
Height (mean ± SD)	1.59 ± 0.06	1.60 ± 0.06	0.284
Weight (mean ± SD)	60.30 ± 8.15	59.82 ± 7.54	0.737
BMI (mean ± SD)	23.97 ± 3.03	23.40 ± 2.31	0.241
< 18.5 (%)	1 (1.67)	2 (3.33)	> 0.9999
18.5–23.9 (%)	32 (53.33)	34 (56.67)	0.714
24–27.9 (%)	18 (30.00)	20 (33.33)	0.695
≥ 28kg (%)	9 (15.00)	4 (6.67)	0.142
Comorbid condition (%)			
Osteoporosis	17 (28.33)		
Hypertension	15 (25.00)		
Sides of KOA (%)			
Left side	18 (30.00)		
Right side	23 (38.33)		
Bilateral	19 (31.67)		
K-L grading scale (%)			
K-L = 1	9 (15.00)		
K-L = 2	22 (36.67)		
K-L = 3	18 (30.00)		
K-L = 4	11 (18.33)		
WOMAC score (mean ± SD)			
Total score	48.92 ± 25.62		
Pain score	13.78 ± 6.11		
Stiffness score	6.43 ± 2.83		
Physical function score	28.52 ± 17.21		

Table 2 Distributions of plasma ccf-mtDNA content by host characteristics in cases and controls

Variables	ccf-mtDNA: Median (Quartile range)		
	KOA ($n = 60$)	HC ($n = 60$)	P^a value
Overall	2.44 (1.10–3.79)	1.08 (0.52–2.12)	**< 0.0001**
Age (years)			
≤ 60.1	2.86 (1.26–3.72)	1.21 (0.59–2.16)	**0.000**
> 60.1	1.88 (0.90–4.66)	0.87 (0.43–2.00)	**0.008**
P^b value	0.318	0.346	
Gender			
Female	2.63 (1.04–3.92)	1.21 (0.52–2.32)	**0.000**
Male	1.98 (1.17–2.96)	0.70 (0.46–1.45)	**0.002**
P^b value	0.526	0.135	
Height (m)			
≤ 1.59	2.86 (1.11–4.42)	1.47 (0.49–2.59)	**0.001**
> 1.59	1.95 (1.03–3.10)	0.80 (0.58–1.12)	**0.001**
P^b value	0.100	**0.043**	
Weight (kg)			
≤ 60	2.69 (1.31–4.18)	1.21 (0.48–2.29)	**0.000**
> 60	1.36 (0.91–3.64)	0.89 (0.59–1.81)	**0.013**
P^b value	0.138	0.527	
BMI (kg/m^2)			
≤ 23.69	2.58 (1.37–4.28)	0.73 (0.45–2.08)	**< 0.0001**
> 23.69	2.09 (0.94–3.54)	1.32 (0.86–2.38)	0.114
P^b value	0.231	0.170	

P^a: Differences between KOA cases and HC

P^b: Differences between the two strata of each host variables

Table 3 Comparison of the general conditions and ccf-mtDNA content among patients with different K-L grades of KOA

K-L n	I 9	II 22	III 18	IV 11	*P* value
Age (mean ± SD)	54.44 (9.52)	60.59 (11.00)	61.56 (9.65)	64.82 (7.14)	0.136
Female (%)	66.67	86.36	72.22	90.91	0.409
Height (mean ± SD)	1.62 (0.06)	1.58 (0.05)	1.59 (0.07)	1.57 (0.04)	0.261
Weight (mean ± SD)	64.44 (13.58)	59.77 (13.58)	59.67 (5.54)	59.00 (7.73)	0.563
BMI (mean ± SD)	24.39 (4.01)	23.95 (2.34)	23.81 (3.27)	23.94 (3.37)	0.975
WOMAC (mean ± SD)					
Total score	18.89 (3.72)	34.18 (9.06)	56.72 (12.18)	90.18 (12.80)	**< 0.0001**
Pain score	7.89 (2.15)	10.14 (2.66)	15.28 (3.21)	23.45 (4.13)	**< 0.0001**
Stiffness score	3.56 (1.59)	4.91 (1.66)	7.28 (1.74)	10.45 (1.64)	**< 0.0001**
Physical function score	7.44 (1.94)	19.14 (6.66)	33.61 (7.46)	56.18 (8.60)	**< 0.0001**
ccf-mtDNA content (median, quartile range)	1.22 (0.84–2.65)	2.26 (0.98–3.12)	3.45 (1.50–4.66)	4.52 (1.09–5.84)	**0.040**

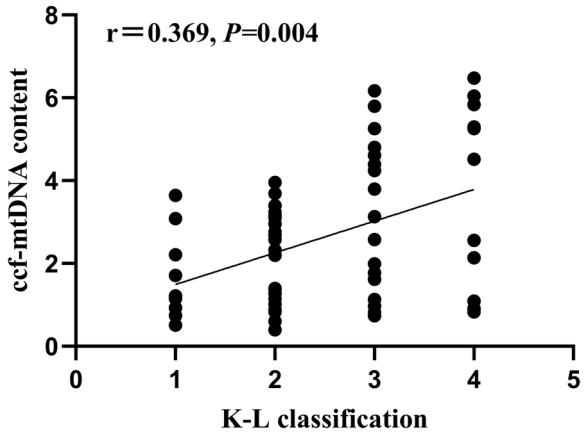

Fig. 1 Correlation of plasma ccf-mtDNA with K-L classification

positively correlated with K-L grading ($r = 0.369$, $P = 0.004$) (Fig. 1). Furthermore, Spearman correlation analysis showed that plasma ccf-mtDNA content was positively correlated with the total WOMAC score, pain score, and joint function score ($r = 0.343$, 0.302, and 0.366; $P = 0.007$, 0.019, and 0.004, respectively); however, no correlation was observed the stiffness score ($r = 0.235$; $P = 0.070$) (Fig. 2).

Comparison of general conditions and ccf-mtDNA content between patients with bilateral KOA and single KOA

Patients with KOA were divided into two groups based on whether the patient had bilateral knee osteoarthritis. The characteristics and expression of ccf-mtDNA were analyzed between the two groups. The results showed that there were no significant differences in age, sex, height, weight, body mass index, or ccf-mtDNA content between the two groups ($P = 0.270$, 0.835, 0.220, 0.755, 0.289, and 0.083, respectively (Table 4).

Association between ccf-mtDNA content and KOA risk

The association between ccf-mtDNA content and KOA risk was modeled using unconditional logistic regression by analyzing ccf-mtDNA content as a categorical variable based on the cut-off value of the median or interquartile distribution of ccf-mtDNA content in the control group. The results showed that patients with KOA who had higher ccf-mtDNA contents (> 1.08) had a significantly increased risk of KOA in both univariate (unadjusted OR, 3.29; 95% CI, 1.50–7.20; $P = 0.003$) and multivariate analyses (adjusted OR, 4.15; 95% CI, 1.71–10.07; $P = 0.002$) compared with those with lower ccf-mtDNA contents (≤ 1.08). In quartile analyses, using patients with the lowest ccf-mtDNA levels as a reference, those with higher ccf-mtDNA levels had a significantly increased risk of KOA in both univariate and multifactorial analyses, with a clear dose-dependent relationship *(P* for trend = 0.001 and < 0.001, respectively) (Table 5).

Association between ccf-mtDNA content and KOA risk stratified by patients' characteristics

We further assessed the association between ccf-mtDNA content and the risk of KOA, stratified by patient characteristics, using multivariate analyses corrected for age, sex, height, weight, and BMI, where appropriate. In the stratified analyses, increased ccf-mtDNA levels significantly increased the risk of developing KOA in all strata, except for patients > 60.1 years ($P = 0.203$), weighing > 60 kg ($P = 0.211$), and those with BMI > 23.69 ($P = 0.355$) (Table 6).

Discussion

In this study, we found that the plasma ccf-mtDNA content was higher in patients with KOA than in HC. In addition, ccf-mtDNA significantly correlated with KOA

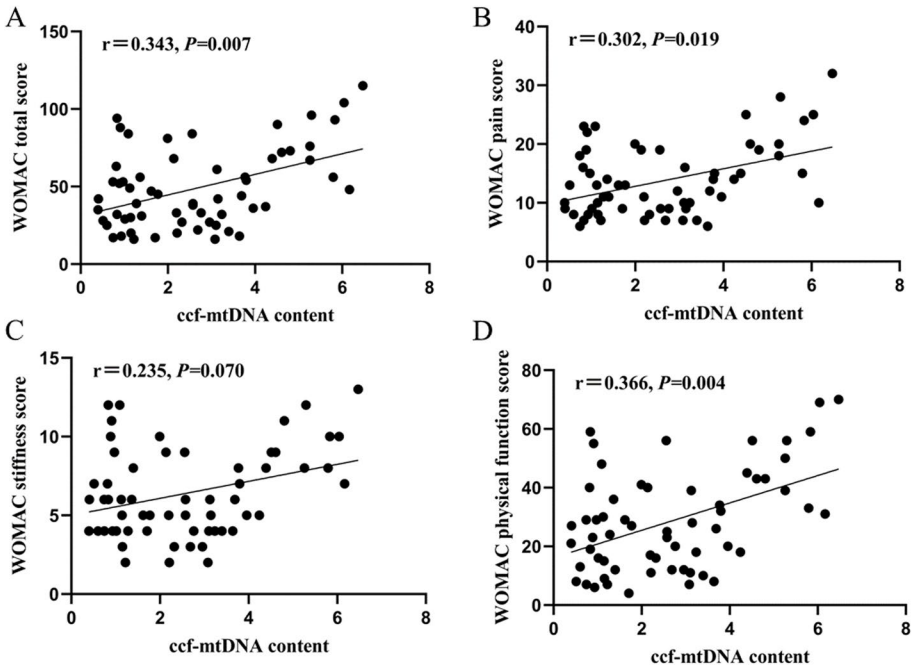

Fig. 2 Correlation of plasma ccf-mtDNA with WOMAC score. **A** Correlation of plasma ccf-mtDNA with WOMAC total score. **B** Correlation of plasma ccf-mtDNA with WOMAC pain score. **C** Correlation of plasma ccf-mtDNA with WOMAC stiffness score. **D** Correlation of plasma ccf-mtDNA with WOMAC physical function score

severity based on the Spearman analysis of the correlation of ccf-mtDNA levels with the X-ray KL classification and WOMAC scores. Logistic regression analysis revealed that ccf-mtDNA may be a risk factor for developing KOA. Therefore, ccf-mtDNA is a marker of mitochondrial dysfunction and cellular stress, and detecting its levels may help prevent and treat KOA.

Consistent with the epidemiological results, we included a high proportion of female participants (80%) in this study, indicating a greater prevalence of KOA among females than males. Additionally, 45% of patients in the KOA group were obese and the age of the patients increased as the K-L grade increased, suggesting that sex, obesity, and age may be risk factors in the pathogenesis of KOA, similar to the findings of a previous study [18].

Notably, the mitochondria do not have the same complex DNA repair mechanisms as the nucleus, and the absence of protective histones in mtDNA is responsible for the damage. Subsequently, the damaged mtDNA fragments are released into the cytoplasm or extracellular space [19, 20]. Following the release into the extracellular space, mtDNA fragments such as ccf-mtDNA can be detected in the blood or other body fluid samples [21, 22]. ccf-mtDNA behaves similarly to DAMPs by activating toll-like receptor 9 (TLR9), inflammatory vesicles, the interferon gene stimulator pathway, and the stimulator pathway to induce inflammation [23]. Increasing evidence suggests that innate response pathways may significantly influence the onset and progression of KOA, particularly TLR [24–26]. Reports revealed that TLR-9 activates nuclear transcription factor-κB (NF-κB) transcription factors, leading to the production of pro-inflammatory cytokines, thereby promoting the development of KOA [27]. Mitochondrial dysfunction maintains an active regenerative cycle through oxidative stress, increased ROS, and mtDNA damage, which are regarded as hallmarks of chronic degenerative diseases such as KOA. The accumulation of ROS and mtDNA damage can activate the NF-κB pathway, which majorly regulates inflammation [28]. Significant increases in ccf-mtDNA levels have been observed in diseases with chronic inflammatory states, such as trauma, sepsis, aging, cancer, and immune-mediated diseases [29–33]. In this study, we found that plasma ccf-mtDNA levels were significantly higher in patients with KOA than in HC. However, our findings are not consistent with those of Panagopoulou et al. Panagopoulou et al. did not find a significant difference in plasma ccf-mtDNA levels between patients with KOA and those of HC ($P=0.852$) [34]. This inconsistency could be attributed to several factors. In their study, the comparison of ccf-mtDNA between patients with KOA and HC was a secondary analysis that did not strictly control for major confounders. Additionally, the study by Panagopoulou et al. had a small sample size, which may

Table 4 Comparison of general conditions and ccf-mtDNA content between patients with bilateral KOA and single KOA

Variables	Bilateral KOA ($n=19$)	Single KOA ($n=41$)	*P* value
Age (mean ± SD)	62.84 (11.50)	59.76 (9.23)	0.270
Female (%)	78.95	80.49	0.835
Height (mean ± SD)	1.57 (0.06)	1.59 (0.06)	0.220
Weight (mean ± SD)	60.79 (8.18)	60.07 (8.23)	0.755
BMI (mean ± SD)	24.59 (3.10)	23.69 (2.99)	0.289
ccf-mtDNA content (median, quartile range)	3.69 (0.91–5.26)	2.14 (1.11–3.18)	0.083

Table 5 Association of plasma ccf-mtDNA content with the risk of KOA

			Unadjusted		Adjusted	
ccf-mtDNA	KOA	HC	OR (95% CI)	*P* value	OR (95% CI)	*P* value
By median						
Lowe	14	30				
Higher	46	30	3.29 (1.50-7.20)	**0.003**	4.15 (1.71-10.07)	**0.002**
By quartile						
1st quartile	3	15	1	-	1	-
2nd quartile	11	15	3.67 (0.85-15.85)	0.082	3.35 (0.76-14.84)	0.112
3rd quartile	12	15	4.00 (0.93-17.12)	0.062	4.49 (0.99-20.47)	0.052
4th quartile	34	15	11.33 (2.85-45.05)	**0.001**	13.36 (3.18-56.19)	**0.000**
P for trend				**0.001**		**< 0.001**

Adjusted for age, gender, height, weight, and BMI

have led to unstable estimates. Finally, this inconsistency could also be due to other factors, such as differences in assay methods (TaqMan method vs. SYBR Green method) or patient ethnicity (Chinese vs. Greek). Hence, further large, well-controlled studies are required to validate these results.

Furthermore, we categorized patients with KOA into four groups following the X-ray KL classification criteria based on the severity of KOA imaging. Additionally, we used WOMAC to assess the severity of symptoms in these patients. Märtens et al. performed a study on the correlation of radiographic changes and pain sensation in shoulder osteoarthritis with patient age. The results show that age was positively correlated with the X-ray K-L classification; the higher the K-L classification, the older the age [35]. Similarly, in this study, we found that as the radiographic K-L grade increased, the age of patients also tended to increase. Furthermore, the WOMAC pain, stiffness, joint function, and total scores were significantly higher as the K-L grade increased, which suggests that the severity of KOA imaging findings is associated with the severity of its symptoms. However, further studies revealed that plasma ccf-mtDNA levels increased sequentially from KOA I–IV groups, suggesting that ccf-mtDNA may be involved in the development of KOA. Notably, joint pain, stiffness, and dysfunction are common symptoms of KOA that affect and quality of life of patients with KOA [36]. Spearman correlation analysis showed that plasma ccf-mtDNA positively correlated with the K-L classification, WOMAC total score, pain score, and joint function score; however, no correlation was found with the stiffness score, suggesting that plasma ccf-mtDNA levels could reflect the severity of KOA. Meanwhile, we analyzed the number of affected joints, and our results showed that ccf-mtDNA content did not significantly differ between patients with bilateral KOA and those with a single KOA. However, ccf-mtDNA content in patients with bilateral KOA tends to be higher than that in patients with single KOA, which may be attributed to the small sample size; hence partially masking the correlation between ccf-mtDNA and the K-L grade. Therefore, future studies with larger sample sizes are needed to investigate the relationship between ccf-mtDNA and the number of affected joints in patients with KOA.

In the early stage of KOA, before irreversible structural alterations occur in the joints, several biomarkers in blood already show alterations. These include inflammatory indicators such as C-reactive protein and interleukin-6 (IL-6), cartilage metabolism indicators, including

Table 6 Association of plasma ccf-mtDNA content with KOA risk stratified by host characteristics

Variables	ccf-mtDNA	KOA	HC	OR (95% CI)	*P* value
Age					
≤ 60.1	Lower	5	15	1	**0.005**
	Higher	29	21	7.49 (1.83–30.70)	
> 60.1	Lower	9	15	1	0.203
	Higher	17	9	2.31 (0.64–8.42)	
Gender					
Female	Lower	12	22	1	**0.012**
	Higher	36	26	3.63 (1.33–9.92)	
Male	Lower	2	8	1	**0.030**
	Higher	10	4	9.76 (1.25–76.10)	
Height					
≤ 1.59	Lower	9	14	1	**0.035**
	Higher	29	24	3.94 (1.10–14.10)	
> 1.59	Lower	5	16	1	**0.004**
	Higher	17	6	8.84 (2.04–38.36)	
Weight					
≤ 60	Lower	7	15	1	**0.008**
	Higher	30	21	6.29 (1.62–24.47)	
> 60	Lower	7	15	1	0.211
	Higher	16	9	2.39 (0.61–9.31)	
BMI					
≤ 23.69	Lower	6	20	1	**0.001**
	Higher	26	15	8.34 (2.27–30.70)	
> 23.69	Lower	8	10	1	0.355
	Higher	20	15	1.88 (0.49–7.18)	

Adjusted for age, gender, height, weight, and BMI where appropriate

type II collagen carboxy-terminal telopeptide and cartilage oligomeric matrix protein, lipid metabolism indicators such as cholesterol and fatty acids, and circulating nucleic acids such as microRNAs and cfDNA [37–39]. Detection biomarkers in plasma reflecting the severity of the disease can predict the destruction of bone and cartilage at an earlier stage, help to further understand the pathogenesis of KOA, and provide a new research direction for the treatment of the disease. The higher number of mtDNA copies makes it easier to detect mtDNA content than nuclear DNAs in body fluid samples, such as plasma and serum, where the total DNA concentration is deficient [40]. In addition, ccf-mtDNA as a liquid biopsy biomarker has the advantages of noninvasiveness and repeated sampling [41]. Therefore, detection of abnormal changes in ccf-mtDNA has become an increasingly important tool for early disease diagnosis. However, plasma ccf-mtDNA is a non-specific marker, as it is significantly elevated in other inflammatory immune diseases, mitochondrial diseases, and tumors [42–44]. Because most KOA patients are older, they are likely to have some associated comorbidities, which may lead to increased ccf-mtDNA levels. Therefore, future studies need larger sample sizes to control for confounding effects of other diseases through multivariate analysis. The irreversible nature of cartilage damage in the knee joints of patients with KOA makes it difficult to treat, and the etiology and pathogenesis of the disease have not yet been clarified. Therefore, it is clinically important to control the risk factors to reduce morbidity. Studies have shown that age, sex, and obesity are risk factors for KOA [45]. In this study, plasma ccf-mtDNA was found to be a pathogenic factor for KOA. In addition to the risk factors reported in previous studies, attention should be paid to patients with elevated plasma ccf-mtDNA levels so that timely measures can be taken to treat KOA.

In stratified analyses, we found that plasma ccf-mtDNA levels were significantly higher in patients with KOA; however, plasma ccf-mtDNA levels greatly differed between patients with KOA and HC, patients with age > 60.1, weight > 60, or BMI > 23.69 compared with patients with age ≤ 60.1, weight ≤ 60, or BMI ≤ 23.69. The close association between age, obesity, and KOA may have masked the relatively modest association conferred by a greater amount of ccf-mtDNA. Furthermore, because of the lack of statistical validity, we cannot exclude the possibility that a smaller number of participants may have contributed to these observations. Therefore, larger independent studies are needed to further explore the interaction between ccf-mtDNA and key demographic variables associated with KOA risk.

Previous findings suggest that ccf-mtDNA may contribute to inflammation and, therefore, may be directly involved in the pathogenesis of KOA. mtDNA activates TLR9 in the endolysosomal membrane when its fragments are released extracellularly and persist in the extracellular fluid, such as ccf-mtDNA. Therefore, activating NF-κB and the transcription of pro-inflammatory genes [46]. Notably, hydroxychloroquine has potential as a disease-modifying osteoarthritis drug (DMOAD) for treating KOA owing to its inhibitory effect on TLR signaling and ability to promote its degradation via a pro-inflammatory pathway [47]. Recent studies have shown that the cGAS-STING pathway may be activated by cytoplasmic mtDNA, and its activation by mtDNA leads to the expression of tumor necrosis factor-alpha (TNF-α) and IL-6, cytokines that are therapeutic targets for KOA [42, 47].

The present study has some limitations. First, only the plasma levels of ccf-mtDNA were measured in this study, whereas the levels of ccf-mtDNA in the joint fluid were not; therefore, the correlation between ccf-mtDNA and KOA in the joint fluid remains unclear. Second, this study was affected by many potential confounding factors.

Future studies with large sample sizes, varying patient-level factors, and controlling for confounding factors are needed. Additionally, patients with KOA undergo surgical or exercise therapy shortly after admission, so we cannot determine the progression of their disease and, therefore, cannot detect the progression of these patients through analysis of baseline ccf-mtDNA. Hence, future community studies are needed to investigate the association between plasma ccf-mtDNA and the progression of KOA in patients. Finally, the study's design was cross-sectional, which did not allow us to conclude a causal relationship between ccf-mtDNA and KOA development. More prospective cohort studies are needed to further confirm this finding.

Conclusion

In summary, ccf-mtDNA was highly expressed in the plasma of patients with KOA, and the plasma ccf-mtDNA level significantly correlated with the severity of KOA, which is a risk factor for the development of KOA. The detection of ccf-mtDNA may provide clues for the prevention and treatment of KOA, and its pro-inflammatory pathway may become a pharmacological target; however, its specific mechanism requires further study.

Abbreviations

KOA	Knee osteoarthritis
ROS	Reactive oxygen species
mtDNA	Mitochondrial DNA
ccf-mtDNA	Circulating cell-free mitochondrial DNA
DAMPs	Damage-associated molecular patterns
HC	Healthy controls
BMI	Body mass index
K-L	Kellgren-Lawrence
WOMAC	Western Ontario and McMaster Universities Osteoarthritis Index
ccf-DNA	Circulating cell-free DNA
qRT-PCR	Quantitative real-time polymerase chain reaction
SD	Standard deviation
OR	Odds ratio
95% CI	95% Confidence intervals
TLR	Toll-like receptor
NF-κB	Nuclear transcription factor-κB
DMOAD	Disease-modifying osteoarthritis drug
TNF-α	Tumor necrosis factor-alpha
IL-6	Interleukin-6

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Authors' contributions

Yan-lin Wu collected and analyzed the data and wrote the manuscript. Shaogui Wan supervised the study and revised the manuscript. Long and Ye used the K-L and WOMAC scores to assess knee injury severity in patients with KOA. Yang and Luo collected blood samples from patients with KOA and HC and isolated plasma samples. Zhong and Xiao extracted ccf-DNA. Chen et al. used qRT-PCR to detect ccf-mtDNA content. Mao-Yuan Wang conceived and designed the study.

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Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request at: wmy.gmu.kf@gmail.com.

Declarations

Ethics approval and consent to participate

The study was conducted following the principles of the Declaration of Helsinki. This study was approved by the Ethics Committee of the First Affiliated Hospital of Gannan Medical University (approval number: LLSC2023-156) and informed consent was obtained from each participant.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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