Urinary mitochondrial DNA may be useful in diagnosing early diabetic nephropathy

LI XUE*, XUE YANG*, YUANYUAN SONG, CAN WANG, JUNJIE ZHOU and HONGYAN LIANG

Department of Clinical Laboratory, The Fourth Affiliated Hospital of Harbin Medical University, Harbin, Heilongjiang 150001, P.R. China

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Abstract. The present study aimed to determine whether urinary mitochondrial (mt)DNA could be combined as a non-invasive biomarker with other clinical findings of kidney injury to help diagnose early diabetic nephropathy (DN). A total of 165 patients with type 2 diabetes mellitus (T2DM) were enrolled in the present study and the mtDNA levels in urine were measured using quantitative PCR. The diagnostic value of urinary mtDNA levels in patients with T2DM was compared using estimated glomerular filtration rate (eGFR) or albumin-to-creatinine ratio staging. Spearman correlation analysis was used to analyze the correlation between urinary mtDNA and other clinical findings. Correlation factors for early DN were assessed using univariate logistic regression analysis. Urinary leukocyte and glucose levels do not interfere with urinary mtDNA levels. In patients with T2DM, the level of urinary mtDNA increases in the early stages of kidney injury and further increases with the severity of kidney injury. Urinary mtDNA levels in patients with eGFR 60-90 ml/min/1.73 m^2 were higher than that in patients with eGFR >90 ml/min/1.73 m^2. The levels of urinary mt89DNA and mt349DNA were negatively correlated with the eGFR level (ρ=-0.437; P<0.001; ρ=-0.390; P<0.001) and positively correlated with the level of cystatin C (ρ=0.177; P=0.025; ρ=0.144; P=0.070). Urinary mtDNA is positively correlated with early DN occurrence [odds ratio (OR), 1.330; 95% confidence interval (CI), 1.175-1.507; P<0.001; OR, 1.328; 95% CI, 1.156-1.525; P<0.001]. In conclusion, urinary mtDNA combined with other clinical indicators of kidney injury may help the diagnosis of early DN.

Correspondence to: Professor Hongyan Liang, Department of Clinical Laboratory, The Fourth Affiliated Hospital of Harbin Medical University, 37 Yi Yuan Street, Harbin, Heilongjiang 150001, P.R. China.
E-mail: aileenliang2005@163.com

*Contributed equally

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Introduction

Diabetic nephropathy (DN) has a high morbidity and mortality rate worldwide, and it is the most prevalent contributor to end-stage renal disease (ESRD) (1). In China, the present authors have identified an increasing number of patients with non-dialysis-dependent chronic kidney disease (CKD) who have diabetes, as assessed through analysis of data retrieved from the Hospital Quality Monitoring System (2). DN has become the leading cause of ESRD, exceeding glomerulonephritis (3). In addition, CKD is the twelfth leading cause of disease mortality (4) and another study showed that co-payments are a particular problem for patients with CKD, leading them to discontinue the medication or stop the dialysis treatment (5). Therefore, the development of biomarkers is significant for the early diagnosis and delayed progression of DN, which can reduce the number of patients with ESRD (6,7) and even eliminate the need for alternative treatments in some patients, reducing suffering and improving quality of life and prognosis.

The kidney is the organ with the second highest oxygen consumption; therefore, it is extremely sensitive to mitochondrial dysfunction (8,9). Renal hypoxia may play an essential role in the progression of DN and it emerged as a common cause of several renal diseases in previous studies (10,11). The mitochondrial (mt)DNA has a critical function in regulating the progression of DN (12). Under oxidative stress due to excess reactive oxygen species (ROS), the mtDNA is not protected by histones and it lacks repair mechanisms throughout almost the entire coding region, making it highly susceptible to damage and mutations (13). The excessive production of ROS that is harmful to mtDNA may also impair the function of the mitochondrial electron transport chain and cause mitochondrial dysfunction, among other issues (8,14). Numerous cells may be affected by diabetes-induced mitochondrial damage, including renal tubular cells that contain abundant mitochondria. DN is a complex disease that is still not fully understood, but mitochondrial impairment is undoubtedly one of the critical causes in the development of DN, regardless of its underlying causes (8-11).

The presence of mtDNA in the urine may be due to its release from renal parenchymal cells into the urine after kidney injury (15). Urinary mtDNA may indicate kidney damage, including DN (16) and non-diabetic kidney

disease (17). In addition, urine samples can be easily collected using non-invasive procedures and have the advantage of large sample volumes (18). Therefore, the present authors intended to investigate whether mtDNA in urine can be used as a non-invasive biomarker to help diagnose early DN.

Materials and methods

Study subjects. The present study enrolled a total of 165 patients with type 2 diabetes mellitus (T2DM), including 48 patients with T2DM without DN, who were admitted to the Fourth Affiliated Hospital of Harbin Medical University between August 2021 and October 2021. Patients with T2DM but without DN had no elevated indicators of any abnormal kidney injury. The degree of renal impairment in patients with T2DM was grouped according to the estimated glomerular filtration rate (eGFR) or albumin-to-creatinine ratio (ACR). The renal impairment was defined as the eGFR <90 ml/min/1.73 m^2 or ACR ≥300 mg/g.

The three groups were classified according to clinical eGFR staging: eGFR ≥90, eGFR 60-89 and eGFR ≤59 ml/min/1.73 m^2. Three groups were classified according to clinical ACR staging: ACR <30, ACR 30-299 and ACR ≥300 mg/g.

Patients with T2DM met the 1999 World Health Organization diagnostic criteria for DM (19). The aforementioned criteria exclude other serious diseases such as malignancy, infections, infectious diseases, pregnancy and medications that affect glucose or albuminuria. Also excluded were different types of diabetic and non-diabetic nephropathy.

The eGFR was estimated using the Cockcroft-Gault (CG) formula: [140-age (years) x body weight (kg)]/creatinine (μmol/l) x0.85 (if female) ml/min/1.73 m^2).

ACR was calculated using the formula: ACR=mAlb/Ucr (mg/g).

The present study conformed to the provisions of the Declaration of Helsinki and was approved by the Clinical Medical Research Ethics Committee of the Fourth Affiliated Hospital of Harbin Medical University (2022-WZYSLLSC-30). All patients gave written informed consent for urine and blood collection and the analysis was approved by the ethic committee.

Data collection. General information regarding all the study subjects was collected, including age and sex. In addition, BMI (weight, kg; height, m), systolic blood pressure (SBP) and diastolic blood pressure (DBP) were also measured.

Sample collection. A blood sample (4 ml) was collected using venipuncture and patients were fasting for >8 h before the measurement of fasting glucose (FPG; Glucose-hexokinase activity detection kit, Beckman Coulter, Inc.) and morning urine specimens were collected from the same patient on the same day. Other biochemical analyses included blood urea (Urea), uric acid (UA), cystatin C (Cys-C) and serum creatinine (Scr) were measured in blood samples [Beckman Coulter Uric Acid and Urea reagents (Beckman Coulter, Inc.); CysC and Scr assay kits (Medconn Diagnostics)]. Glycated hemoglobin (HbA1c) was measured in blood samples (Bio-Rad CDM; Bio-Rad Laboratories, Inc.). Urine samples were collected for routine examination of urine creatinine (Ucr), microalbumin (mAlb) (Beckman AU5800; Beckman Coulter, Inc.) and urinary sediment (DIRUI FUS-2000; Dirui Industrial Co., Ltd.).

Urinary mtDNA preparation. Urine samples were centrifuged at 7,584 x g for 10 min at 4˚C; then, the supernatant was separated, frozen and kept at -80˚C until further analysis. Purified mtDNA was isolated from 200 μl urine supernatant using Nucleic Acid Extraction Kit (magnetic bead method; Zybio, Inc.), according to the manufacturer's instructions. Subsequently, the mtDNA fragments were detected using the LightCycler® 480 real-time PCR detection system [Roche Diagnostics (Shanghai) Co., Ltd.]. A standard curve from 10^1 to 10^4 copies/ml was created using a 10x serial dilution of a standard sample [purified 400 base pairs (bp) mitochondrial clones (accession number of sequence >OR062 595.1); Sangon Biotech Co., Ltd.] as a positive quantification reference. Each sample was analyzed three times, the results were averaged and the corresponding concentration was extrapolated from the standard calibration curve. Finally, the ratio of urinary mtDNA was normalized to its corresponding Ucr for each subject to quantify the urinary mtDNA of each individual. Amplification reactions contained a total volume of 20 μl including 10 μl 2X Ace Taq Master Mix (Vazyme Biotech Co., Ltd.), 1 μl of EvaGreen (Biotium, Inc.), 0.6 μl of forward and reverse primers (Sangon Biotech, Co., Ltd.), 3.4 μl ddH_2O and 5 μl mtDNA. The amplification reactions of mt89DNA primers were carried out at 95˚C for 5 min, followed by 40 cycles at 95˚C for 20 sec, 56˚C for 20 sec and 72˚C for 20 sec. The amplification reactions of mt349DNA primers were carried out at 95˚C for 5 min, followed by 45 cycles at 95˚C for 30 sec, 54˚C for 30 sec, and 72˚C for 30 sec. To assess the expression of mtDNA in urine, the results were expressed as mtDNA activity equivalent to 1 g of creatinine (U/g Ucr) (20). Primer sequences are shown in Table I.

Statistical analysis. The software used for statistical analysis was SPSS version 25.0 (IBM Corp.) and GraphPad Prism version 9.0 (GraphPad Software; Dotmatics). Normally distributed data were expressed as mean ± standard deviation, while variables with skewed distribution were expressed as median (interquartile range). Comparisons between more than two groups were made using the Kruskal-Wallis test followed by Dunn's multiple comparisons test or a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Spearman correlation analysis was used to determine the correlation of other clinical variables. Factors correlated with early DN were assessed by univariate logistic regression analysis and multivariate logistic regression analysis comparing urinary mtDNA with other clinical variables. P<0.05 was considered to indicate a statistically significant difference.

Results

Patients' baseline characteristics. patients with T2DM were divided into three groups according to eGFR: eGFR≥90, eGFR 60-89 and eGFR≤59 ml/min/1.73 m^2 groups. The basic clinical characteristics of the three groups are summarized in Table II. As renal function decreased, eGFR decreased due

Table I. mt89DNA and mt349DNA primer sequences

Primer	Sequence	Length	Number of sequence
mt89 upstream	5'-CCTTACCACGCTACTCCTACC-3'	89 bp	>OR062595.1
mt89 downstream	5'-GCTTTGAAGGCTCTTGGTCTG-3'		
mt349 upstream	5'-AAGAGCCTTCAAAGCCCTCAG-3'	349 bp	>OR062595.1
mt349 downstream	5'-TGGCTGAGTGAAGCATTGGAC-3'		

mt, mitochondrial DNA.

Table II. Baseline clinical characteristics in patients with T2DM in different eGFR groups.

	eGFR (ml/min/1.73 m^2)			
Parameter	≥90 (n=116)	60-89 (n=34)	≤59 (n=15)	P-value
Age (years)	52.54±9.69[a]	66.47±6.96	71.73±9.92	<0.001
Sex				
Male	32	18	8	
Female	84	16	7	
BMI (kg/m^2)	26.87±3.46[b]	24.08±2.47[a]	24.62±3.15	<0.001
SBP (mm Hg)	136.88±19.97[a,b]	146.47±20.63	153.33±20.31	0.002
DBP (mm Hg)	86.65±12.57[b]	81.12±10.48	80.80±15.59	0.032
eGFR (ml/min/1.73 m^2)	123.01 (105.26-148.70)[a,b]	76.69 (67.49-81.08)[a]	48.36 (20.87-53.67)	<0.001
FPG (mmol/l)	8.04 (6.58-10.55)	7.03 (5.70-8.97)	7.96 (4.56-10.38)	0.097
HbA1c (%)	9.00 (7.50-10.40)	7.85 (7.20-9.33)	7.85 (6.93-9.05)	0.022
mAlb (mg/l)	11.95 (7.09-33.43)	12.10 (4.89-46.88)	596.00 (3.61-1,190.00)	0.074
Ucr (mmol/l)	9.00 (5.74-11.95)[a,b]	5.73 (3.98-7.76)	4.34 (2.82-6.23)	<0.001
ACR (mg/g)	12.10 (6.43-35.25)[a]	16.70 (7.75-59.08)[a]	1,231.20 (15.50-3,480.30)	0.005
Scr (μmol/l)	61.25 (54.78-70.55)[a,b]	68.60 (59.15-83.33)[a]	109.80 (80.00-369.10)	<0.001
Urea (mmol/l)	5.49 (4.84-6.35)[a]	5.73 (4.82-6.93)[a]	8.72 (7.46-16.39)	<0.001
UA (μmol/l)	331.9 (272.23-401.13)	314.25 (282.03-375.45)	393.20 (328.20-491.20)	0.096
Cys-C (mg/l)	0.92 (0.79-1.04)[a]	0.97 (0.84-1.14)[a]	1.74 (1.28-2.94)	<0.001

Data were presented as mean ± SD or as median (IQR) when they were not normally distributed. Overall comparison by the Mann Whitney test. P<0.05 indicated statistical significance. [a]P<0.05 was considered significant compared with eGFR <60 (ml/min/1.73 m^2) [b]P<0.05 was considered significant compared with eGFR 60-89 (ml/min/1.73 m^2), [a]P<0.05 was considered significant compared with eGFR <60 (ml/min/1.73 m^2). BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; FBG, fasting blood-glucose; HbA1c, glycated hemoglobin; mAlb, microalbumin; Ucr, urine creatinine; ACR, albumin-creatinine ratio; Scr, serum creatinine; UA, uric acid; Cys-C, cystatin.

to glomerular impairment. ACR increased due to elevated mAlb and decreased Ucr. Scr, Urea and Cys-C increased due to decreased filtration. Age was higher in patients with poorer renal function, accompanied by an increase in SBP.

Urinary leukocytes and urinary glucose do not interfere with urinary mtDNA levels. Fresh urine from patients with T2DM but without DN was collected for qualitative testing of urinary leukocytes and glucose levels. According to the test strips, the urinary leukocyte positive degree was divided into - to 3+ and urinary glucose positive degree was divided into - to 4+. In patients with T2DM but without DN, there was no significant difference in urinary mtDNA levels, indicating that urinary leukocytes and urinary glucose did not affect urinary mtDNA levels (P>0.05; Fig. 1A-F). Comparisons were performed using one-way ANOVA followed by Tukey's post hoc test.

Elevated urinary mtDNA levels in patients with T2DM with mild kidney injury. To study the change in mtDNA level, the degree of renal impairment in patients with T2DM was grouped by clinical eGFR staging. The levels of urinary mt89DNA and mt349DNA at eGFR 60-89 ml/min/1.73 m^2 [0.56 (0.42-0.84) x10^9 and 0.45 (0.32-0.74) x10^9 copies/g, respectively] were higher than those at eGFR ≥90 [0.37 (0.27-0.55) x10^9 copies/g and 0.29 (0.21-0.47) x10^9 copies/g, respectively] ml/min/1.73 m^2 (P=0.005 and P=0.015, respectively), which showed an increase in urinary mtDNA levels during the mild kidney injury.

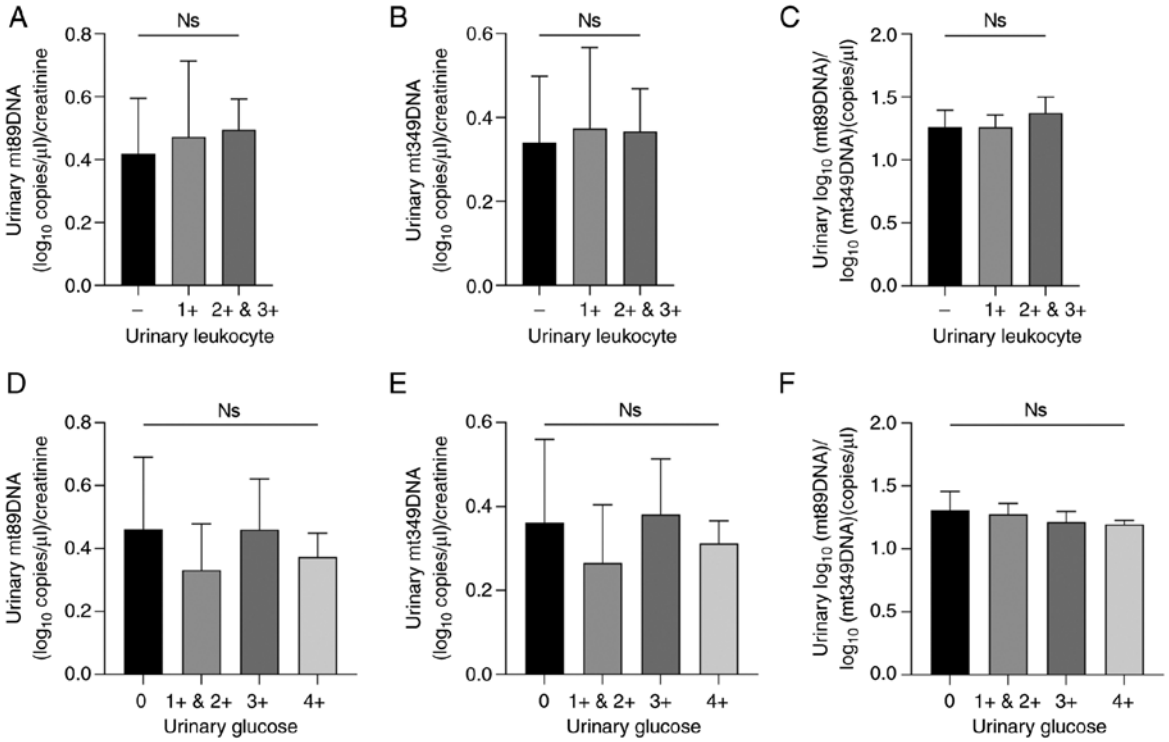

Figure 1. Urinary leukocytes and urinary glucose do not interfere with urinary mtDNA levels. (A) Urinary mt89DNA/creatinine ratio levels compared in different urinary leukocyte positive degrees; P=0.650. (B) Urinary mt349DNA/creatinine ratio levels compared in different urinary leukocyte positive degrees; P=0.852. (C) Urinary mt89DNA/mt349DNA ratio levels compared in different urinary leukocyte positive degrees; P=0.344. (D) Urinary mt89DNA/creatinine ratio levels compared in different urinary glucose positive degrees; P=0.585. (E) Urinary mt349DNA /creatinine ratio levels compared in different urinary glucose positive degrees; P=0.646. (F) Urinary mt89DNA/mt349DNA ratio levels compared in different urinary glucose positive degrees; P=0.078. Data are presented as mean ± standard deviation. Comparisons were performed using one-way ANOVA followed by Tukey's post hoc test. mtDNA, mitochondrial DNA; Ns, non-significant.

Levels of urinary mt89DNA and mt349DNA at eGFR ≤59 ml/min/1.73 m^2 [0.92 (0.64-1.61)x10^9 and 0.68 (0.46-1.20) x10^9 copies/g, respectively] were higher than those at eGFR ≥90 ml/min/1.73 m^2 (P<0.001 for both). The levels of urinary mt89DNA and mt349DNA at eGFR ≤59 ml/min/1.73 m^2 were higher than those at eGFR 60-89 ml/min/1.73 m^2 (P<0.017 and P<0.025, respectively). The aforementioned results suggest that urinary mtDNA levels further increase with the severity of kidney injury (Fig. 2A and B). The ratio of mt89DNA to mt349 in urine was not significantly different in different groups [1.23 (1.18-1.30)x10^9 vs. 1.26 (1.21-1.36) x10^9 vs. 1.34 (1.21-1.39)x10^9 copies/g, respectively] (Fig. 2C).

When patients with T2DM were grouped according to clinical ACR staging, urinary mt89DNA levels were higher in ACR ≥300 mg/g [0.93 (0.36-1.19)x10^9 copies/g] than those in ACR <30 mg/g [0.42 (0.27-0.60)x10^9 copies/g] and ACR 30-299 mg/g [0.52 (0.36-0.71)x10^9 copies/g; P=0.001 and P<0.037, respectively]. Urinary mt349DNA levels were higher in ACR ≥300 mg/g [0.64 (0.27-0.83)x10^9 copies/g] than those in ACR <30 mg/g [0.33 (0.21-0.49)x10^9 copies/g; P=0.004; Fig. 2D and E]. The ratio of urinary mt89DNA to mt349 was higher in ACR ≥300 mg/g than that in ACR <30 mg/g [1.33 (1.23-1.40)x10^9 vs. 1.23 (1.19-1.30)x10^9 copies/g; P=0.038; Fig. 2F]. Comparisons were performed using the Kruskal-Wallis test followed by Dunn's multiple comparisons test.

Correlation of urinary mtDNA with clinical variables in patients with T2DM. Urinary mtDNA expression and clinical variables in patients with T2DM were correlated using Spearman correlation analysis (Tables III and IV). Urinary mt89DNA was negatively correlated with eGFR (ρ=-0.437; P<0.001) and positively correlated with Cys-C (ρ=0.177; P=0.025). Urinary mt349DNA was negatively correlated with eGFR (ρ=-0.390; P<0.001).

Urinary mtDNA is a correlating factor for early DN. The present study compared urinary mtDNA and clinical variables between patients with T2DM in the eGFR ≥90 ml/min/1.73 m^2 group and the eGFR 60-89 ml/min/1.73 m^2 group to analyze correlated factors for early DN.

Through univariate logistic regression analysis, it was found that the occurrence of early DN was positively associated with urinary mt89DNA levels [odds ratio (OR), 1.330; 95% confidence interval (CI), 1.175-1.507; P<0.001], positively associated with mAlb and Urea levels (OR, 1.002; 95% CI, 1.001-1.003; P=0.003 and OR, 1.418; 95% CI, 1.142-1.760; P=0.002), and positively correlated with Cys-C levels (OR, 1.372; 95% CI, 1.179-1.597; P<0.001). After multivariate adjustments, the occurrence of early DN was positively associated with urinary mt89DNA (OR, 1.322; 95% CI, 1.152-1.516; P<0.001), and positively correlated with Cys-C levels (OR, 1.319; 95% CI, 1.081-1.608; P=0.006; Table V).

Based on univariate logistic regression analysis, it was found that the occurrence of early DN was positively associated with urinary mt349DNA levels (OR, 1.328; 95% CI, 1.156-1.525; P<0.001), positively associated with mAlb and

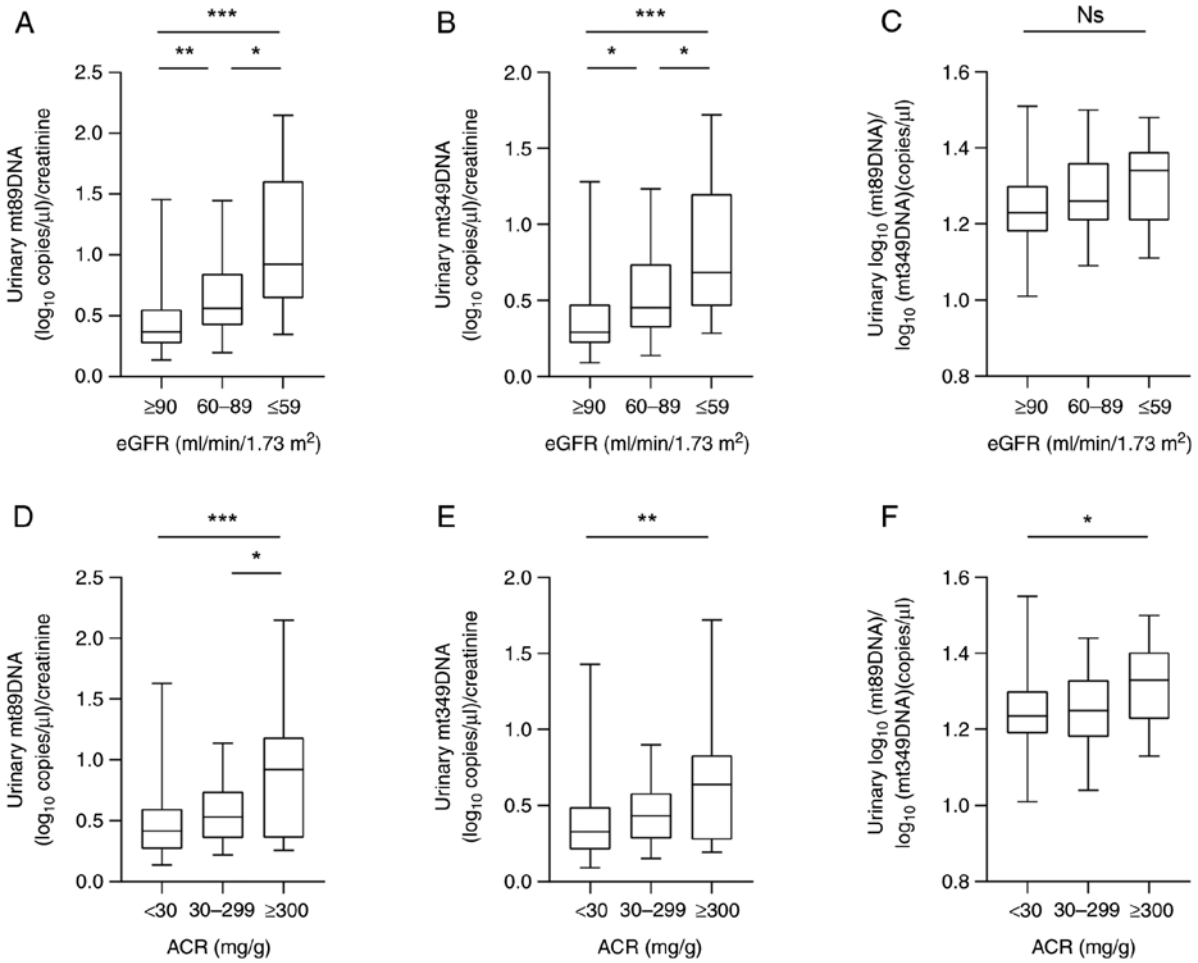

Figure 2. Increased urinary mtDNA levels in patients with T2DM with mild kidney injury. (A) Urinary mt89DNA levels in patients with T2DM were staged based on eGFR, with eGFR ≥90 (n=116), eGFR 60-89 (n=34) and eGFR ≤59 ml/min/1.73 m^2 (n=15). (B) Urinary mt349DNA levels in patients with T2DM were staged based on eGFR. (C) Comparison of the ratio of mt89DNA to mt349 levels in the urine of patients with T2DM according to eGFR staging. (D) Urinary mt89DNA levels in patients with T2DM were staged based on ACR, with ACR ≥300 (n=111), ACR 30-299 (n=36) and ACR <30 mg/g (n=18). (E) Urinary mt349DNA levels in patients with T2DM were staged based on ACR. Data are presented as median with inter-quartile range. (F) Comparison of the ratio of mt89DNA to mt349 levels in the urine of patients with T2DM according to ACR staging. Comparisons were performed using the Kruskal-Wallis test followed by Dunn's multiple comparisons test. *P<0.05, **P<0.01 and ***P<0.001. mtDNA, mitochondrial DNA; T2DM, type 2 diabetes mellitus; eGFR, estimated glomerular filtration rate; ACR, albumin-to-creatinine ratio; Ns, non-significant.

Table III. Univariate correlation between urinary mt89DNA (log transformed data before analysis) and clinical variables.

Clinical variable	ρ correlation coefficient	P-value
eGFR (ml/min/1.73 m^2)	-0.437	<0.001
mAlb (mg/l)	-0.070	0.374
Scr (μmol/l)	-0.023	0.773
Urea (mmol/l)	0.082	0.297
UA (μmol/l)	-0.098	0.213
Cys-C (mg/l)	0.177	0.025

Spearman correlation analysis. eGFR, estimated glomerular filtration rate; mAlb, microalbumin; Scr, serum creatinine; UA, uric acid; Cys-C, cystatin.

Table IV. Univariate correlation between urinary mt349DNA (log transformed data before analysis) and clinical variables.

Clinical variable	ρ correlation coefficient	P-value
eGFR (ml/min/1.73 m^2)	-0.390	<0.001
mAlb (mg/l)	-0.101	0.199
Scr (μmol/l)	-0.049	0.534
Urea (mmol/l)	0.060	0.448
UA (μmol/l)	-0.112	0.156
Cys-C (mg/l)	0.144	0.070

Spearman correlation analysis. eGFR, estimated glomerular filtration rate; mAlb, microalbumin; Scr, serum creatinine; UA, uric acid; Cys-C, cystatin.

Urea levels (OR, 1.002; 95% CI, 1.001-1.003; P=0.003 and OR, 1.418; 95% CI, 1.142-1.760; P=0.002) and Cys-C levels were positively correlated (OR, 1.372; 95% CI, 1.179-1.597; P<0.001). After multivariate adjustments, the occurrence of

Table V. Correlated factors for eGFR ≥90 (ml/min/1.73 m^2) and eGFR <90 (ml/min/1.73 m^2) were analyzed using binary logistic regression.

	Univariate		Multivariate	
Clinical variables	OR (95% CI)	P-value	OR (95% CI)	P-value
mt89DNA (per 10^8 copies/g)	1.330 (1.175-1.507)	<0.001	1.322 (1.152-1.516)	<0.001
mAlb (per 1 mg/l)	1.002 (1.001-1.003)	0.003	1.000 (0.999-1.001)	0.938
Urea (per 1 mmol/l)	1.418 (1.142-1.760)	0.002	1.208 (0.898-1.624)	0.213
UA (per 1 μmol/l)	1.000 (0.997-1.004)	0.923	-	-
Cys-C (per 10 mg/l)	1.372 (1.179-1.597)	<0.001	1.319 (1.081-1.608)	0.006

Values express as odds ratios (OR) and 95% confidence interval (CI). eGFR, estimated glomerular filtration rate; mt, mitochondrial DNA; mAlb, microalbumin; UA, uric acid; Cys-C, cystatin.

Table VI. correlate factors for eGFR ≥90 (ml/min/1.73 m^2) and eGFR <90 (ml/min/1.73 m^2) were analyzed using binary logistic regression.

	Univariate		Multivariate	
Clinical variables	OR (95% CI)	P-value	OR (95% CI)	P-value
mt349DNA (per 10^8 copies/g)	1.328 (1.156-1.525)	<0.001	1.337 (1.145-1.560)	0.002
mAlb (per 1 mg/l)	1.002 (1.001-1.003)	0.003	1.000 (0.999-1.001)	0.979
Urea (per 1 mmol/l)	1.418 (1.142-1.760)	0.002	1.208 (0.898-1.625)	0.212
UA (per 1 μmol/l)	1.000 (0.997-1.004)	0.923	-	-
Cys-C (per 10 mg/l)	1.372 (1.179-1.597)	<0.001	1.335 (1.097-1.626)	0.004

Values express as odds ratios (OR) and 95% confidence interval (CI). eGFR, estimated glomerular filtration rate; mt, mitochondrial DNA; mAlb, microalbumin; UA, uric acid; Cys-C, cystatin.

early DN was positively correlated with urinary mt349DNA levels (OR, 1.337; 95% CI, 1.145-1.560; P=0.002), and positively correlated with Cys-C levels (OR, 1.335; 95% CI, 1.097-1.626; P=0.004; Table VI).

Discussion

Usually, DN is diagnosed several years after the onset of the disease, during which various organ damage has occurred. On the other hand, the early diagnosis of DN provides the possibility of renal protective therapy, which can significantly delay the progression to ESRD (21,22).

Currently, the typical clinical indicators used to assess the diagnosis and progression of DN are Scr, eGFR, Cys-C and mAlb (23). mAlb is known to be the earliest marker of DN. However, mAlb is nonspecific and is involved in exercise, urinary tract injury and acute disease (11,24-26). In cases where a patient refuses to undergo a kidney biopsy, the DN diagnosis requires a combination of several non-invasive indicators to be accurate. Therefore, there is a need to explore new non-invasive biomarkers that would benefit DN patients (23,27,28).

A previous study reported that mitochondrial dysfunction can affect key cellular functions (29). The kidney is an organ rich in mitochondria and damage to the parenchymal cells of the kidney can lead to mtDNA leakage into the urine (15,30). Cell free DNA (cfDNA), refers to highly fragmented DNA that is present in the human blood circulation and is free from cells. As an ultra-noninvasive tool for liquid biopsy, urinary cfDNA has unique advantages in molecular profiling of tumors, which is considered to have a complementary and synergistic effect on serum and plasma in diagnosis, progression surveillance, treatment monitoring and prognosis for both urological and non-urological cancers (31). The present study used urine as a biomarker because it is simple to extract and can be screened in patients (32).

Alterations in mitochondria may be the underlying cause of various complex diseases. A previous study implicated mtDNA in triggering and maintaining inflammation in the heart (33). mtDNA in urine was linked to chronic obstructive pulmonary disease (30). Urinary mtDNA may serve as a potential marker for bladder cancer (34). In addition, studies have associated mitochondrial disorders with some kidney diseases. For example, the prognosis of non-diabetic chronic kidney disease is associated with urinary mtDNA (35). Another study showed that urinary mtDNA can be used to assess renal mitochondrial damage in patients with vascular hypertension undergoing renal revascularization (36). However, to the best of the authors' knowledge, there are only a few reports on patients with DN related to urinary mtDNA.

The size of cfDNA is ~40-bp. Longer DNA fragments >200 bp or even >10 kbp are derived from necrotic cells. Shorter DNA fragments <100 bp result from apoptotic cells, including circulating tumor-specific cell-free DNA, mtDNA and bacterial DNA (37). Therefore, two mtDNA primers with different lengths, namely mt89DNA and mt349DNA, were chosen in the present study to compare the changes of mtDNA levels following kidney damage caused by DN. Although a small amount of DNA is theoretically released by leukocytes in the urine, urine specimens collected in the present study were centrifuged to exclude the effect of most leukocytes and the experimental results showed that urinary leukocytes did not affect the urinary mtDNA levels. This result suggests that urinary mtDNA may originate more from renal cell damage than from leukocytes. In addition, the results of the current study showed that urinary glucose levels also did not affect urinary mtDNA levels. In a previous study from the present authors, the expression of circulating mt89DNA and mt349DNA was not specific and was detected at low levels, suggesting that the mtDNA in the urine mainly originated from kidney injury than from circulation (38). However, this finding needs to be further demonstrated through renal pathology biopsy.

It has been shown that high plasma glucose leads to mitochondrial dysfunction through the activation of several metabolic pathways (8) and oxidative stress caused by damage to the kidney itself, which leads to mtDNA damage. Therefore, mtDNA expression is different in different degrees of kidney injury (39).

The present study showed that the mtDNA levels in the urine were already elevated in case of mild kidney injury. As the degree of renal injury progressed, mtDNA levels increased further. mtDNA ratios increased with the degree of renal damage (Fig. 2). These results may indicate the predominance of apoptotic forms of kidney injury. Furthermore, when kidney injury is moderate or severe, apoptotic cells are produced in large numbers, leading to a significant release of small fragments of mtDNA. Even though mtDNA in urine correlated weakly with other indicators of kidney injury, it correlated negatively with eGFR, suggesting that mtDNA levels in urine are related to kidney function (Tables III and IV).

An important change in early DN reaching the later stages of DN is a decrease in eGFR. Thus, the present study compared urinary mtDNA and clinical variables in patients with T2DM in the eGFR ≥90 and eGFR <90 ml/min/1.73 m^2 groups to analyze correlated factors for early DN. The results showed that the occurrence of early DN was positive correlated with urinary mtDNA, mAlb, Urea and Cys-C levels following univariate logistic regression analysis, and only positive correlated with urinary mtDNA and Cys-C levels following multivariate adjustment. This finding may provide new clues for the diagnosis of early DN; using clinical variables in combination with urinary mtDNA is useful (Tables V and VI).

Preventing or improving mitochondrial dysfunction can prevent and treat DN (10). Mitochondrial damage is involved in the pathological process of DN and the use of antioxidants as therapeutics will provide a way for the clinical treatment of DN (40).

The diagnosis of early DN is challenging. Most patients do not undergo renal biopsy but rely more on the combined analysis of non-invasive biomarkers of each renal injury, which is more reliable for the diagnosis of early DN (3). In patients with T2DM, urinary mtDNA is associated with renal impairment and urinary mtDNA is one of the factors associated with early DN (38). Therefore, the detection of urinary mtDNA is useful for the diagnosis of early DN.

There are several shortcomings to the present study. First, the sample size was insufficient, especially for patients with severe kidney injury. Second, it would have been more convincing if long-term follow-ups were started from the early stages of the disease and kidney biopsy samples collected from the patients with T2DM to assess the role of urinary mtDNA in DN progression. Third, the age of subjects in healthy control group were generally lower than that in type 2 diabetic patients. The present study found that the level of mtDNA in type 2 diabetes patients without kidney damage was higher compared with that in the healthy control group (data not shown), which may be due to oxidative stress caused by diabetes leading to mitochondrial damage, leading to an increase in the level of mtDNA. However, due to differences in age, the results may be biased, so we did not present this result in this article and only made a preliminary comparison. Therefore, the present findings need to be verified by further studies. Animal experiments will be performed to verify the correlation of mtDNA in DN.

Although urine mtDNA cannot be used as a specific marker for DN in the clinic, it is clearly correlated with DN. The kidney is the second organ with the highest oxygen consumption in the body and is rich in mitochondria (8,9). mtDNA release is caused by early cell injury and the early development of DN can be caused by mitochondrial damage such as oxidative stress in kidney cells thus affecting the production of urinary mtDNA (12,13). When other influencing factors are excluded, urinary mtDNA combined with other clinical indicators of kidney injury may help the diagnosis of early DN (Table V and VI). Injury-released mtDNA is involved in the occurrence and development of T2DM. In patients with T2DM, urinary mtDNA increases in the early stages of DN and urinary mtDNA is one of the factors associated with early DN; therefore, it can be used as a non-invasive biomarker and combined with other clinical indicators of kidney injury to help diagnose early DN.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HL designed the study. LX and XY interpreted results and drafted the manuscript. YS tested urinary mtDNA in the samples. JZ and CW collected samples, and were involved in the acquisition and analysis of data. HL and LX confirm the

authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study conforms to the provisions of the Declaration of Helsinki and was approved by the Clinical Medical Research Ethics Committee of the Fourth Affiliated Hospital of Harbin Medical University (approval no. 2022-WZYSLLSC-30). All patients gave written informed consent for urine and blood collection and the analysis was approved by the ethic committee.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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