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Decreased Peripheral Mitochondrial DNA Copy Number is Associated with the Risk of Heart Failure and Long-term Outcomes

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Abstract: Mitochondrial DNA (mtDNA) copy number variation (CNV), which reflects the oxidant-induced cell damage, has been observed in a wide range of human diseases. However, whether it correlates with heart failure, which is closely related to oxidative stress, has never been elucidated before. We aimed to systematically investigate the associations between leukocyte mtDNA CNV and heart failure risk and prognosis.

A total of 1700 hospitalized patients with heart failure and 1700 age- and sex-matched community population were consecutively enrolled in this observational study, as well as 1638 (96.4%) patients were followed prospectively for a median of 17 months (12–24 months). The relative mtDNA copy number of leukocyte of peripheral blood or cardiac tissue was measured in triplicate by quantitative real-time PCR method.

Patients with heart failure possessed much lower relative mtDNA copy number compared with control subjects (median 0.83, interquartile range [IQR] 0.60–1.16 vs median 1.00, IQR 0.47–2.20; $P < 0.001$), especially for the patients with ischemic etiology (median, 0.77 for ischemic and 0.91 for non-ischemic, $P < 0.001$). Patients with lower mtDNA copy number exhibited 1.7 times higher risk of heart failure (odds ratio 1.71, 95% confidence interval [CI] 1.48–1.97, $P < 0.001$). Long-term follow-up (median of 17 months) showed that decreased mtDNA copy number was significant associated with both increased cardiovascular deaths (hazard ratio [HR] 1.58, 95% CI 1.16–2.16, $P = 0.004$) and cardiovascular rehospitalization (HR 1.48, 95% CI 1.21–1.82, $P < 0.001$). After adjusting for the conventional risk factors and medications, lower mtDNA copy numbers were still significantly associated with 50% higher cardiovascular mortality ($P = 0.035$).

In conclusion, mtDNA copy number depletion is an independent risk factor for heart failure and predicts higher cardiovascular mortality in patients with heart failure.

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Abbreviations: CI = confidence intervals, CNV = copy number variation, DCFH-DA = Dichlorodihydrofluorescein Diacetate, DHE = Dihydroethidium, HDL-C = high-density lipoprotein cholesterol, HF = heart failure, HR = hazard ratio, IQR = interquartile range, LDL-C = low-density lipoprotein cholesterol, MtDNA = mitochondrial DNA, NT-proBNP = N-terminal prohormone of brain natriuretic peptide, NYHA = New York Heart Association, OR = odds ratio, ROS = reactive oxygen species, TC = total cholesterol, TG = triglycerides.

INTRODUCTION

Heart failure (HF), which is a complex disturbance at end-stage of a variety of cardiovascular diseases, has become a major burden to the health care system all around the world.[1,2] Despite the large medical costs associated with HF and the improvements in therapy, the absolute mortality for HF remains approximately 50% within 5 years after diagnosis.[3,4] It remains critical to identify novel risk factors and biomarkers that could lead to novel treatment approaches, primary prevention, and risk stratification.[5]

Over the past several decades, clinical data and animal experimental studies have provided substantial evidences that oxidative stress, which can cause cellular dysfunction including lipid peroxidation and DNA damage, is enhanced in state of heart failure.[6–10] Mitochondria, the key role of which is to generate cellular ATP through oxidative phosphorylation, are not only the great source of intracellular reactive oxygen species (ROS), but also the major targets of ROS-mediated damage.[11–13] Accumulating evidences have suggested that mitochondrial dysfunction plays a critical role in the development of HF.[14,15]

Human mitochondria DNA (mtDNA) genome is an approximately 16.5K base-pair circular double-stranded DNA molecule that encodes 13 polypeptides of the respiratory chain, 22 transfer RNAs, and 2 ribosomal RNAs.[16,17] Compared with nuclear DNA, mtDNA is more susceptible to oxidative attack ascribed to its poor repair capacity and lacking of histone-like proteins.[18] To date, mtDNA copy number variation of peripheral blood mononuclear cells has been observed in different kinds of human cancers, which are considered to have a close relationship with oxidative stress.[19–23] However, whether mtDNA copy number of peripheral blood cells is altered under chronic heart failure, also a term of ROS-related disease, has not

been elucidated yet. In the present study, we enrolled 1700 HF patients and 1700 age-, and sex-matched normal controls to explore the association of the peripheral leukocyte mtDNA content, also termed mtDNA copy number, with HF risk and prospectively assess the prognostic value in term of cardiovascular mortality and event of cardiovascular rehospitalization.

METHODS

Patients and Control Cohort

Seventeen hundred patients with chronic HF were consecutively enrolled from inpatients of Cardiovascular Division of Tongji Hospital (Wuhan, China) between January 2008 and August 2014. At the time of study entry, detailed clinical data were obtained with standardized questionnaires administered to the patients, with verification via medical records. The diagnosis of HF was confirmed based on the medical histories, physical examinations, laboratory tests, echocardiography, and according to the established protocols and criteria of the American College of Cardiology/American Heart Association Guideline.[24] The severity of HF was categorized by New York Heart Association (NYHA) functional class criteria. The patients with following conditions were excluded: acute infection, severe liver dysfunction (alanine aminotrasferase >5 upper limit of normal), renal dysfunction (serum creatinine >220 mmol/L), or a history of malignancy. The follow-up information was obtained from a subset of 1638 (96.4%) patients during a median period of 17 months (interquartile range [IQR] 12–24 months) by telephone or face-to-face interviews after the patients were discharged and the medicine treatments were also recorded at the same time. The primary end point was death from cardiovascular causes, and second end point is event of first cardiovascular rehospitalization defined as visits to the hospital for angina-, arrhythmias-, or heart failure-related symptoms such as dyspnea and edema. The patients lost from follow-up were excluded for survival analysis because of an incomplete dataset.

A total of 1700 control subjects were frequency matched to the patients by age, sex, ethnicity, and county of residence. The inclusion criterion for the control group was absence of any known cardiovascular disease. Comorbidities such as hypertension, hyperlipidemia, diabetes, and smoking were not treated as exclusion criteria. All control subjects were also asked for a detailed medical history and received a careful physical examination.

The study was designed according to the ethical principles of the Declaration of Helsinki. All protocols and methods were approved by the ethics committees of the local hospital (Ethics Committees of Tongji Hospital). Written informed consents were obtained from all participants.

MtDNA Determination in Circulating Leukocytes and Heart Tissues

Blood samples were collected on day 1 of hospitalization without medical treatments. Leukocytes were separated from the whole blood sample collected in K2-EDTA tubes, and total genomic DNA was extracted using the QG-Mini80 workflow with a DB-S kit (FUJIFILM Corporation, Tokyo, Japan) according to the manufacturer's instructions. In the subgroup of 10 patients who accepted heart transplantations, we obtained both the heart tissues of end-stage heart failure and blood samples. DNAs of these heart tissues were extracted by the QIAamp DNA Mini Kit (No. 51304, QIAGEN, Germany) according to

the instructions. The relative mtDNA copy number, defined as the copy number ratio of a mitochondrial gene (*ND1*) to a single-copy nuclear gene (*HBB*, human β-globins gene) compared with a same calibrator DNA, was assayed in triplicate by a quantitative real-time PCR (q-PCR) method based on the 384-well ABI 7900HT TaqMan platform (Applied Biosystems, Foster City, CA). Specific primers and probes used to amplify the mt-*DN1* gene and the nuclear *HBB* gene were as follows: *ND1*: F 5'-GACGCCATAAAACTCTTCACCAA, R 5'-AGG TTGAGGTTGACCAGGGG, probe 5'-FAM-CCATCACCCT CTACATCACCGCCC-TAMRA; *HBB*: F 5'-CTGGGCATGT GGAGACAGAGAAGACT, R 5'-AGGCCATCACTAAAGG-CACCGAGC, probe 5'-FAM- CCCTTAGGCTGCTGGTGG TCTACCCTT -TAMRA. PCR reactions were performed using standardized threshold for TaqMan without knowledge of clinical data. For every sample, we amplify the 2 genes in the same well position of separate 2 plates to avoid position effect. The calibrator sample was measured in every plate to standardize the variation among different runs. Standard curves derived from a serially diluted DNA (100.00–1.56 ng, 2-fold dilution; 7 points) were run for both the mt-*ND1* gene and the *HBB* gene, with good linearity ($R^2 > 0.99$ for both). The corresponding efficiency (E) for each primers/probe set was calculated according to the equation: $E = e^{[-1/slope]}$ (Figure S1, http://links.lww.com/MD/A892) and the coefficients of variation were 2.7% and 4.8% for *ND1* gene and *HBB* gene, respectively.

ROS Assay in Myocardium and Peripheral Blood Cells

Samples of heart tissues were collected and immediately stored at −80°C. ROS were quantified in the heart tissues of left ventricle from end-stage heart failure patients or healthy donors died in traffic accidents. Dihydroethidium (DHE) staining was performed to evaluate ROS generation according to the manufacturer's instructions (Beyotime Institute of Biotechnology, Shanghai, China). Peripheral lymphocytes from HF patients and control subjects were prepared using lymphocyte separation medium (LTS10770125, Tianjin Hao Yang Biological Manufacture, Tianjin, China). Intracellular ROS was detected by flow cytometry using Dichlorodihydro fluorescein Diacetate (DCFH-DA), which was diluted to a final concentration of 10 mmol/L, added and incubated with the cells for 30 minutes at 37°C in the dark as previously reported (Beyotime Institute of Biotechnology, Shanghai, China).[25] The relative levels of fluorescence were quantified by a FACS Calibur 440E flow cytometer (Becton Dickinson, SanJose, CA).

Statistical Analysis

The sample size was calculated based on the interim analysis for the primary end point and we got a power of 0.96 to achieve the odds ratio (OR) of 1.7 for the risk of HF.

The clinical baseline characteristics of HF patients and control subjects were presented as mean ± SD, median (interquartile range [IQR]), or No. (frequency) where appropriate. Comparisons between 2 groups were performed by independent samples *t* test for continuous variables and χ^2 test for categorical variables. Variables with skewed distribution such as the levels of N-terminal prohormone of brain natriuretic peptide (NT-proBNP) and the relative mtDNA copy number were compared by nonparametric Mann-Whitney *U* test and made natural log-transformation before included in the

regression models. The correlations analyses between the relative mtDNA copy number and the clinical characteristics were performed using the standard liner-regression models or Pearson rank coefficients after natural-log transformation treatments. The independent effect of relative mtDNA copy number on heart failure risk was tested using unconditional logistic regression models. Kaplan-Meier curves and Cox proportional hazards regression models were established to evaluate the associations of mtDNA copy number with the adverse outcomes of patients with heart failure. The 95% confidence intervals (CIs) were computed from regression parameters. Three Cox-regression models were set up as follows: without adjustment; adjusting for age, sex, and conventional clinical risk factors such as smoking, hypertension, hyperlipidemia, and diabetes; adjusting for all covariates in model 2 plus NYHA functional class, Ischemic etiology, NT-proBNP level, and medications. In the regression models, the mtDNA copy number was analyzed as categorical variables based on a cutoff point at the median values in the controls or continuous variables, respectively.

Correlation of mitochondrial DNA content between leukocytes of peripheral blood and cardiomyocytes was evaluated by comparative Ct method and Pearson correlation coefficient was calculated after natural logarithmic transformation.

Statistical analyses were performed using SPSS statistical package (Version 17.0, SPSS Inc., Chicago, IL) or Prism 5.0 (GraphPad Software Inc, San Diego, CA). All *P* values reported are 2-sided, and $P < 0.05$ was considered to be statistically significant.

RESULTS

Baseline Characteristics

The characteristics of all the HF patients and matched control subjects were summarized in Table 1. The patients and the control subjects were well matched at age (57.9 ± 13.4 vs 57.7 ± 11.0; $P = 0.596$) and sex (65.6% vs 65.6% male; $P = 1.00$). There are no differences in the blood pressure levels between the two groups, but the heart rate was significantly higher for HF patients (83.3 ± 20.3 vs 73.3 ± 10.3, $P < 0.001$). The patients had a significantly higher percentage of smoking, hypertension, hyperlipidemia, and diabetes histories (all $P < 0.001$). Compared with the nonischemic HF group (n = 910), patients with ischemic etiology (n = 790) seemed to be older (62.6 ± 10.4 vs 53.8 ± 14.3, $P < 0.001$), had a higher percentage rate of male sex (68.7% vs 62.9%, $P = 0.011$), smokers ($P = 0.003$), medical histories including hypertension ($P < 0.001$), hyperlipidemia ($P < 0.001$), and diabetes ($P < 0.001$), and significantly lower levels of diastolic blood pressure ($P < 0.001$), heart rate ($P < 0.001$), and plasma NT-proBNP level ($P < 0.001$).

The Relative mtDNA Copy Number Determinants in Community Controls and HF Patients

To clarify the clinical significance of alterations in mtDNA copy number, its associations with different basic clinical parameters were analyzed by multiple linear regressions both in community controls and HF patients. In control subjects, the relative mtDNA copy number increased steadily with age at a

TABLE 1. Baseline Characteristics of HF and Control Groups

Characteristic	Total HF (n = 1700)	Ischemic HF (n = 790)	Nonischemic HF (n = 910)	Control Group (n = 1700)
Sex, male (%)	1115 (65.6)	543 (68.7)*	572 (62.9)	1115 (65.6)
Age at sample collection, y	57.9 ± 13.4	62.6 ± 10.4*	53.8 ± 14.3	57.7 ± 11.0
Current smoking	666 (39.2)†	339 (42.9)*	327 (35.9)	802 (47.2)
Hypertension	1036 (60.9)†	559 (70.8)*	477 (52.4)	742 (43.6)
Hyperlipidemia	477 (28.1)†	256 (32.4)*	221 (24.3)	339 (19.9)
Diabetes	449 (26.4)†	253 (32.0)*	196 (21.5)	157 (9.2)
Systolic pressure, mmHg	132.0 ± 23.6	131.0 ± 27.3	130.9 ± 27.2	130.0 ± 20.0
Diastolic pressure, mmHg	80.9 ± 16.1	79.2 ± 14.2*	82.3 ± 17.5	80.2 ± 21.2
Heart rate, beats/min	83.3 ± 20.3†	78.5 ± 16.7*	87.2 ± 22.2	73.3 ± 10.3
Total Cholesterol, mg/dL	3.80 (3.16–4.53)†	3.78 (3.13–4.55)	3.81 (3.22–4.52)	4.41 (3.77–5.04)
Triglycerides, mg/dL	1.11 (0.81–1.64)†	1.22 (0.83–1.76)*	1.05 (0.78–1.56)	1.25 (0.89–1.85)
HDL-C, mg/dL	0.92 (0.77–1.13)†	0.92 (0.78–1.10)	0.92 (0.74–1.15)	1.25 (1.03–1.49)
LDL-C, mg/dL	2.26 (1.76–2.88)†	2.18 (1.66–2.88)*	2.34 (1.82–2.88)	2.47 (1.96–3.03)
NT-proBNP, pg/mL	1698 (374–5435)	956 (192–3081)*	2593 (765–6682)	
NYHA functional class				
II	747 (43.8)	441 (55.8)	303 (33.3)	
III	606 (35.6)	242 (30.6)*	364 (40.0)	
IV	350 (20.6)	107 (13.5)	243 (26.7)	
LVEDD, mm	54 (48–63)	50 (46–57)*	59 (51–67)	
LAD, mm	40 (35–46)	38 (34–42)*	43 (37–49)	
Ejection fraction (%)	50 (33–62)	57 (42–65)*	40 (30–58)	

Values are presented as mean ± SD, or number (%), or median (interquartile range). HDL-C = high-density lipoprotein cholesterol, HF = heart failure, LAD = left atrial dimension, LDL-C = low-density lipoprotein cholesterol, LVEDD = left ventricular end-diastolic dimension, NT-proBNP = The N-terminal prohormone of brain natriuretic peptide, NYHA = New York Heart Association.
*$P < 0.05$ compared with the nonischemic HF.
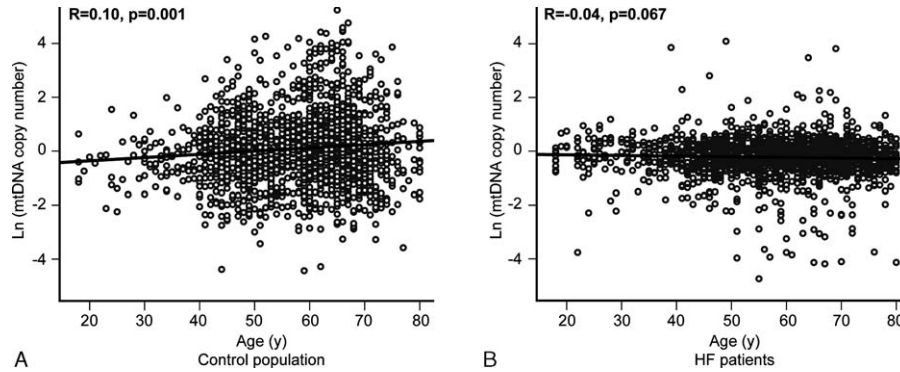†$P < 0.05$ compared with control.

FIGURE 1. Relative mtDNA copy number as a function of age in control subjects and heart failure patients. Relative mtDNA copy number, expressed as the natural log (Ln) of the mtDNA copy number to the nuclear one copy gene ratio, is plotted as a function of age. The open circles represent 1700 controls (A) or 1700 patients (B), and linear regression lines are drawn to elucidate the correlations. Correlation coefficients (*r*) between Ln (relative mtDNA copy number) and age were 0.10 ($P < 0.001$) for controls (A) and -0.04 ($P = 0.067$) for heart failure patients (B). Yearly increase of relative mtDNA copy number was 0.012 in controls and 0.002 in heart patients.

mean yearly rate of 0.012 ± 0.003 ($r = 0.10$, $P = 0.001$), which was vanished in the HF patients (mean rate of 0.002 ± 0.001; $r = -0.04$, $P = 0.067$) (Figure 1). The correlations of mtDNA copy number with the basic characteristics evaluated by univariant regression models were presented in Table S1, http://links.lww.com/MD/A892. In multivariate stepwise regression analysis with forced entry of all the basic parameters involving age, sex, smoking status, medical histories and the levels of systolic blood pressure, diastolic blood pressure, total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C), as shown in Table 2, only age (p < 0.001), smoking ($P < 0.001$), TG ($P = 0.044$) and HDL-C levels ($P < 0.001$) in the control group were still associated with mtDNA copy number. However, except that LDL-C level was significantly associated with mtDNA copy number in the HF population in the full-adjusted models ($P = 0.007$), no other correlations were detected.

In this study, we observed a highly significant lower relative mtDNA copy number in HF patients than controls (median 0.83, IQR 0.60–1.16 vs median 1.00, IQR 0.47–2.20, respectively; $P < 0.001$) (Figure 2A). Interestingly, the patients with ischemic etiology had an even lower level of mtDNA copy number compared with nonischemic patients (0.77 [0.56–1.08] vs 0.91 [0.63–1.22], $P < 0.001$), whereas both of them possessed significantly lower levels compared with the control population (both $P < 0.001$) (Figure 2A). Lower mtDNA copy number presented a tendency related to the higher New York Heart Association functional class, but not statistically significant ($P = 0.071$) (Figure 2B). NT-proBNP levels ($P = 0.053$), echocardiography parameters including left ventricular end-diastolic diameter ($P = 0.988$), and ejection fraction (p = 0.190) were not associated with mtDNA copy number levels of leukocytes (Figure S2, http://links.lww.com/MD/A892).

TABLE 2. The Relative CNV Correlates in the General Population and HF Patients

Correlate	Controls Relative Ratio (Std β)[*],[†]	*P*	HF Patients Relative Ratio (Std β)[*],[†]	*P*
Sex	0.112	<0.001	−0.008	0.802
Age	0.025	0.395	−0.027	0.360
Current smoking	0.092	0.001	−0.044	0.175
Hypertension	0.069	0.058	−0.004	0.915
Hyperlipidemia	−0.013	0.716	0.030	0.436
Diabetes	0.017	0.559	0.050	0.071
Systolic pressure	0.069	0.065	−0.030	0.491
Diastolic pressure	−0.021	0.483	−0.038	0.378
Heart rate	0.043	0.123	0.031	0.297
Total cholesterol	0.078	0.234	−0.094	0.053
Triglycerides	−0.079	0.044	0.065	0.093
HDL-C	−0.227	<0.001	0.048	0.121
LDL-C	−0.073	0.160	0.113	0.007

HDL-C = high-density lipoprotein cholesterol, HF = heart failure, LDL-C = low-density lipoprotein cholesterol.
[*]Standardized β (Std β) reflects the change in the dependent variable for 1-SD change in the independent variable.
[†]In multivariate stepwise regression analysis with forced entry of all the basic parameters shown above.
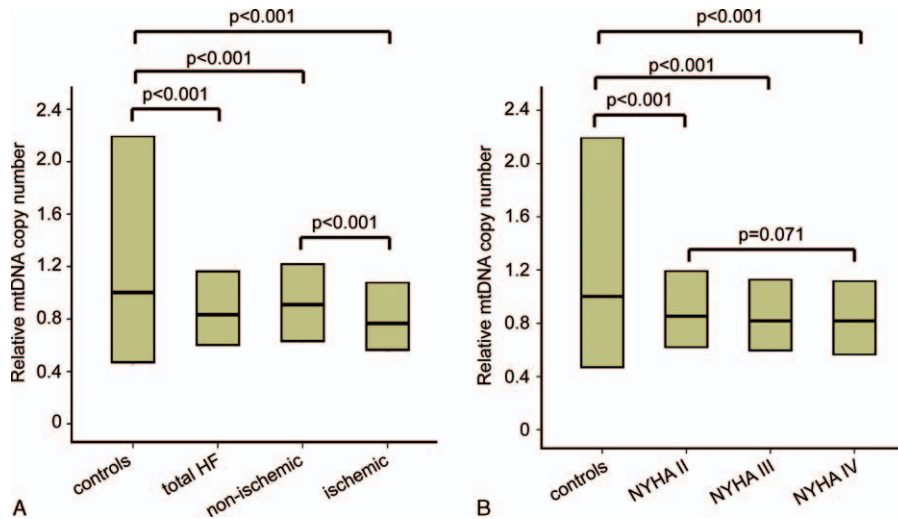
FIGURE 2. The mtDNA copy number distributions in control subjects and heart failure patients. Y axis represents the relative mtDNA copy number. (A) The mtDNA copy number distributions of controls, patients and subgroups additionally stratified by etiologies. (B) The mtDNA copy number distributions stratified by New York Heart Association functional class. Bold black horizontal lines represent the median of relative mtDNA copy number with the top and button lines of the boxes representing the interquartile ranges.

Association of the Relative mtDNA Copy Number With Heart Failure Risk

Unconditional logistic regression analyses were conducted to evaluate the HF risk. Decreased mtDNA copy number was significantly associated with elevated risk of heart failure (OR 1.81, 95% confidence interval [CI] 1.58−2.08, $P < 0.001$). Other clinical factors associated with HF risk were current smoking (OR 1.39, 95% CI 1.21−1.59, $P < 0.001$), hypertension (OR 2.01, 95% CI 1.76−2.31, $P < 0.001$), hyperlipidemia (OR 1.57, 95% CI 1.34−1.84, $P < 0.001$), and diabetes (OR 3.53, 95% CI 2.90−4.29) (Table 3). In the additional multiple analysis adjusted for age, sex, smoking status, hypertension, hyperlipidemia, and diabetes, decreased mtDNA copy number was found to be an independent risk factor for HF (OR 1.71, 95% CI 1.48−1.97, $P < 0.001$ when treated as dichotomous variables and OR 1.30, 95% CI 1.22−1.39, $P < 0.001$ when treated as continuous variables, respectively) (Table 3).

MtDNA Copy Number Variation and Prognosis of Heart Failure

Baseline characteristics with an mtDNA copy number that was higher or lower than the median mtDNA copy number of control population are listed in Table 4. Patients with lower mtDNA copy number seemed to be older (58.6 vs 56.7, $P = 0.008$), had higher level of systolic pressure (133 vs 129 mmHg, $P = 0.004$), smaller left ventricular end-diastolic diameter (50 vs 60 mm, $P = 0.007$), and higher level of ejection fraction (57% vs 40%, $P = 0.016$). Ischemic etiology (51.0% vs 38.8%, $P < 0.001$), histories of hypertension (63.7% vs 56.6%, $P = 0.004$), and diabetes (28.4% vs 23.3%, $P = 0.023$) were also more prevalent in patients with lower mtDNA copy number.

The prognostic value of peripheral relative mtDNA copy number on cardiovascular mortality and rehospitalization were next evaluated in the prospective cohort of 1638 (96.4%) heart failure patients during a median of 17 months' (12−24 months) follow-up time. Totally, 244 patients (14.9%) died during the follow-up, with 214 patients for cardiovascular causes. A total

of 462 patients (28.2%) had cardiovascular rehospitalization events as defined above. Figure 3A portrayed the unadjusted associations between the relative mtDNA copy number and clinical endpoints by the Kaplan-Meier curve method with stratifying the patients according the median value of mtDNA copy number of controls. Lower mtDNA copy number was associated with higher cardiovascular mortality (15.1% vs 9.3%, Log rank $P = 0.003$) and higher risk of cardiovascular rehospitalization (31.7% vs 21.9%, $P < 0.001$). Subgroup analyses were summarized in Figure 3B and the HRs for the

TABLE 3. Logistic Regression of the Clinical Factors and Heart Failure Risk

Variables	OR	95% CI	P
Sex	1.00	0.87−1.15	1.000
Age	1.00	0.99−1.01	0.596
Smoking	1.39	1.21−1.59	<0.001
Hypertension	2.01	1.76−2.31	<0.001
Hyperlipidemia	1.57	1.34−1.84	<0.001
Diabetes	3.53	2.90−4.29	<0.001
Relative mtDNA copy number			
Below median vs above median			
Model 1[§]	1.81	1.58−2.08	<0.001
Model 2[†]	1.71	1.48−1.97	<0.001
1-SD decrease in ln variable[*]			
Model 1[§]	1.33	1.24−1.42	<0.001
Model 2[†]	1.30	1.22−1.39	<0.001

95% CI = 95% confidence interval, OR = odds ratio.
[*]These data have been natural-log transformed as skewed distribution and the odds ratio indicated 1-SD decrease of ln (relative mtDNA copy number).
[†]Adjusted for age, sex, smoking status, hypertension, hyperlipidemia, and diabetes.
[§]Adjusted for age and sex.

TABLE 4. Clinical Characteristics of Heart Failure Patients in the Follow-up Cohort, According to the mtDNA Copy Number

Characteristic	Below Median (n = 1058)	Up Median (n = 580)	*P*
Sex, male (%)	700 (66.2)	379 (65.2)	0.739
Age at sample collection, y	58.6 ± 13.1	56.7 ± 13.9	0.008
Ischemic etiology (%)	540 (51.0)	225 (38.8)	<0.001
NYHA functional class			
II	453 (42.8)	259 (44.7)	
III	378 (35.7)	209 (36.0)	0.567
IV	227 (21.5)	112 (19.3)	
Current smoking	404 (38.2)	236 (40.7)	0.32
Hypertension	674 (63.7)	328 (56.6)	0.004
Hyperlipidemia	295 (27.9)	162 (27.9)	0.983
Diabetes	301 (28.4)	135 (23.3)	0.023
Systolic pressure, mmHg	133.0 ± 26.1	129.1 ± 24.8	0.004
Diastolic pressure, mmHg	81.4 ± 16.4	80.2 ± 15.9	0.149
Heart rate, beats/min	83.1 ± 20.6	84.0 ± 19.7	0.413
Cholesterol, mg/dL	3.79 (3.13−4.55)	3.80 (3.21−4.51)	0.73
Triglycerides, mg/dL	1.21 (0.83−1.75)	1.05 (0.79−1.57)	0.553
HDL-C, mg/dL	0.92 (0.79−1.11)	0.92 (0.74−1.15)	0.722
LDL-C, mg/dL	2.17 (1.66−2.89)	2.34 (1.81−2.88)	0.397
NT-proBNP, pg/mL	963 (191−3278)	2720 (779−6987)	0.289
LVEDD, mm	50 (46−57)	60 (52−67)	0.007
LAD, mm	38 (34−42)	43 (37−49)	0.294
Ejection fraction (%)	57 (42−65)	40 (30−57)	0.016
Digoxin	235 (22.2)	139 (24.0)	0.419
Diuretics	475 (44.9)	251 (43.3)	0.528
ACEI	592 (56.0)	318 (54.8)	0.661
ARB	145 (13.7)	83 (14.3)	0.735
β-blocker	612 (57.8)	321 (55.3)	0.328
Spironolactone	393 (37.1)	219 (37.8)	0.806

Values are presented as mean ± SD, or number (%), or median (interquartile range). ACEI = angiotensin-converting enzyme inhibitors, ARB = angiotensin II receptor blocker, HDL-C = high-density lipoprotein cholesterol, LAD = left atrial diameter, LAD = left atrial dimension, LDL-C = low-density lipoprotein cholesterol, LVEDD = left ventricular end-diastolic dimension, LVEDD = left ventricular left ventricular end-diastolic diameter, NT-proBNP = The N-terminal prohormone of brain natriuretic peptide, NYHA = New York Heart Association.

adverse clinical events were consistent across all prespecified subgroups according to various baseline characteristics. Decreased mtDNA copy number was found to be a significant predictor in the univariant Cox-regression analysis and in a multivariate model after adjustment for conventional clinical covariates and medications for risks of cardiovascular death (adjusted HR = 1.48, 95% CI 1.03−2.14, $P = 0.035$) and rehospitalization (adjusted HR = 1.28, 95% CI 1.01−1.82, $P = 0.046$), respectively. The associations were still significant when mtDNA copy number was treated as a continuous variable (adjusted HR 1.25, $P = 0.023$ for cardiovascular mortality and adjusted HR 1.32, $P < 0.001$ for cardiovascular rehospitalization) (Table 5).

The Correlation Between Peripheral Blood Cells and Cardiomyocytes

To further investigate the correlations between peripheral blood and myocardium, we tested mtDNA/nDNA copy number in both leukocytes and heart tissues of 10 end-stage HF patients who received heart transplantations. Results showed the blood cell mtDNA/nDNA copy number was correlated significantly with that of their cardiomyocytes (Pearson $R = 0.718$,

$P = 0.019$; Figure 4A). ROS productions in end-stage heart tissues and peripheral blood cells, characterized by fluorescence intensity of DHE (for heart tissue) and DCFH-DA (for peripheral lymphocytes) were also detected. Results showed ROS levels were significantly higher in patients with heart failure compared with healthy controls both in heart tissues and peripheral lymphocytes (Figure 4B and C). The clinical characteristics of these participants were summarized in Supplementary S2 and S3, http://links.lww.com/MD/A892.

DISCUSSION

In the present study, we provided the first clinical evidence in a large-scale case/control population demonstrating that depletion of peripheral mtDNA copy number was independently associated with higher risk of HF (OR 1.71, $P < 0.001$) and predicted higher long-term cardiovascular mortality (adjusted HR 1.48, $P = 0.035$).

Substantial evidences have demonstrated that mtDNA copy number, an indicator of mitochondrial biogenesis, is decreased in the human right ventricle during the process through hypertrophy to failure[15] and in the ventricular tissue of both ischemic HF patients and murine models of myocardial
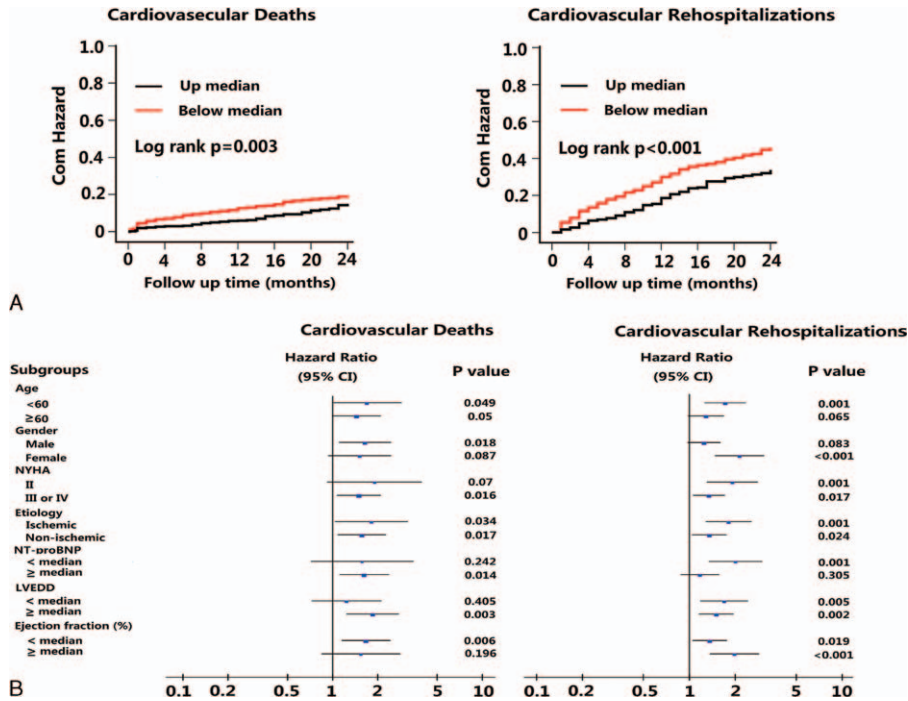
FIGURE 3. Clinical outcomes in the follow-up heart failure population and prespecified subgroups. (A) Kaplan-Meier curves for the primary end point (deaths from cardiovascular causes) and second endpoint (cardiovascular rehospitalization). Red lines represent the patients whose mtDNA copy number levels are below the median level of control population and black lines represent those patients whose copy number are above the median. X axis represents the follow-up time and the Y axis represents cumulative risk. (B) Subgroup analyses of the primary and secondary endpoint according to various baseline characteristics. The position of blue square represents the hazard ratio and the size of the square corresponds to the number of patients in each subgroup. Black lines represent the 95% confidence interval.

infarction.[13,26] In the present study, we observed similar depletion of mtDNA copy number as determined in peripheral leukocytes of patients in the state of heart failure compared with the community control populations. The specific regulation of signaling pathways by ROS is increasingly recognized as an important contributor to the pathophysiology of heart failure.[27] MtDNA is defective in self-repairing, and it is in close proximity to the inner membrane of the mitochondrion and exposed directly to intracellular ROS generated by mitochondrial oxidative respiratory chain.[28] It also has been reported that mtDNA damage could cause dysfunction of the electron transport chain complexes and subsequently lead leads to more ROS production and more damage of mtDNA during heart failure.[29,30] Therefore, oxidative stress-related mitochondrial genome damage might be the possible explanation for the decrement of mtDNA copy number.[10,18]

In parallel with the mtDNA damage caused by excessive ROS generation, the injuries of integral mitochondrion functionality, which is maintained by the mtDNA content, are also reflected by a decline in the mitochondrial RNA (mtRNA) transcripts and synthesis of proteins coding by mtDNA.[13,31,32] An intimate link among ROS, mtDNA damage, and defects in the electron transport function may directly trigger integral signaling of hypertrophy and apoptosis in cardiomyocytes and mediate fibrosis through activating cardiac fibroblasts.[29,33,34] Specifically, ROS can directly impair contractile function by modifying proteins central to excitation-contraction coupling.[10] All of these exacerbate the pathophysiologic processes and affect clinical outcomes of heart failure.

As known, heart failure has a complex pathophysiology and may involve disorders of multiple organs and systems. Systemically increased oxidative stress in tissues and blood play important roles in the pathogenesis of heart failure.[35,36] Previous study reported that intracellular oxidants were significantly increased in the peripheral blood polymorph nuclear leukocytes, monocytes, and lymphocytes of HF patients.[36,37] These findings are consistent with the observations in our study that peripheral blood cells exhibit parallel alterations of mtDNA/nDNA copy number and ROS level with cardiomyocytes. Whereas deficient pulmonary clearance of ROS owing to pulmonary congestion in heart failure is likely the main reason for the high ROS levels of peripheral blood cells.[36] On one hand, mitochondria of circulating blood cells can sense oxidative stress and are subsequently injured reflected by mitochondrial dysfunction, which could further aggravate peripheral ROS generations.[13,38] On the other hand, activated white blood cells with dysfunctional mitochondria may trigger systemic and myocardial ROS production in chronic HF through the inflammation-related mechanisms[39–41]. The cross-talk of peripheral blood and myocardium forms a catastrophic feedback cycle, which results in greater myocardial injury and oxidative stress.

In this study, we highlight the correlation between mtDNA copy number and ischemic heart disease. First, we detected significant association of mtDNA copy number and serum lipid levels both in control population and HF patients (Table 2). Additionally, we found that the patients with an ischemic etiology of heart failure possessed even lower mtDNA copy

TABLE 5. Multivariate-adjusted Analysis of the mtDNA Copy Number to Long-term Clinical Outcomes

	Below Median Vs Above Median			1-SD Decrease of Variable		
	HR	95% CI	*P*	HR*	95% CI*	*P*
Cardiovascular deaths						
Model 1	1.58	1.16−2.16	0.004	1.25	1.07−1.47	0.006
Model 2	1.61	1.18−2.20	0.003	1.27	1.07−1.50	0.006
Model 3	1.48	1.03−2.14	0.035	1.25	1.03−1.51	0.023
Cardiovascular rehospitalisation						
Model 1	1.48	1.21−1.82	<0.001	1.29	1.15−1.44	<0.001
Model 2	1.48	1.20−1.81	<0.001	1.32	1.17−1.48	<0.001
Model 3	1.28	1.01−1.63	0.046	1.32	1.15−1.51	<0.001

CI = confidence interval. Model 1, without adjustment; Model 2 includes covariates of age, sex, smoking, NYHA class and etiology and histories of hypertension, hyperlipidemia, and diabetes; Model 3 includes all covariates of Model 2 + ln (NT-proBNP) + ln (LV) + ln (EF%) + medications.
*HR indicates hazard ratio for per each SD decrease of ln (relative mtDNA copy number).

number compared with the patients with nonischemic heart failure ($P < 0.001$). However, biological evidence in humans for a causal role of mtDNA content in the pathophysiology of chronic HF or atherosclerosis remains to be established.

Additionally, we observed a linear yearly increase in natural log of relative mtDNA copy number ratio (0.012) in the control population, which was diluted in patients as a result of copy number decrement accompanied by disease status. Mitochondria dysfunctions including damage of mtDNA are supposed to be in the process of ageing, but actually, there is no consensual conclusion about the relationship between mtDNA copy number and aging.[42] Some studies reported a tendency of decrease of mtDNA copy number toward age,[43,44] others reported increase,[45] or even no significant change.[22] This may concern the complex balance between oxidative stress and antioxidative defense, and therefore, more powerful evidences are needed in the future to clarify this issue through rigorous methology and mechanism exploration. We did not detect any association between the mtDNA CNV and the clinical parameters reflecting the severity of heart failure such as NYHA functional class, left ventricular ejection fraction, and the level of NT-proBNP, which have been widely considered as important biomarkers to evaluate the severity and prognosis of heart failure in recent years.[46,47] Additionally, in the full model adjusted for the NYHA functional class and NT-proBNP level, the mtDNA copy number is still an independent indicator to predict the cardiovascular mortality. It can be inferred that mtDNA copy number affects the long-term outcome of heart failure through a different mechanism from these conventional biomarkers.

We chose human peripheral leukocytes to study the relationship between the mtDNA content and HF risk, not just because the samples were easy to obtain. What's more meaningful is that we demonstrate a consistent conclusion with what has previously been observed in the failing heart tissues. Given all these observations, human leukocytes are good target cells to reflect the oxidative stress status in heart failure and peripheral mtDNA CNV is a promising noninvasive biomarker for heart failure risk and prognosis prediction.
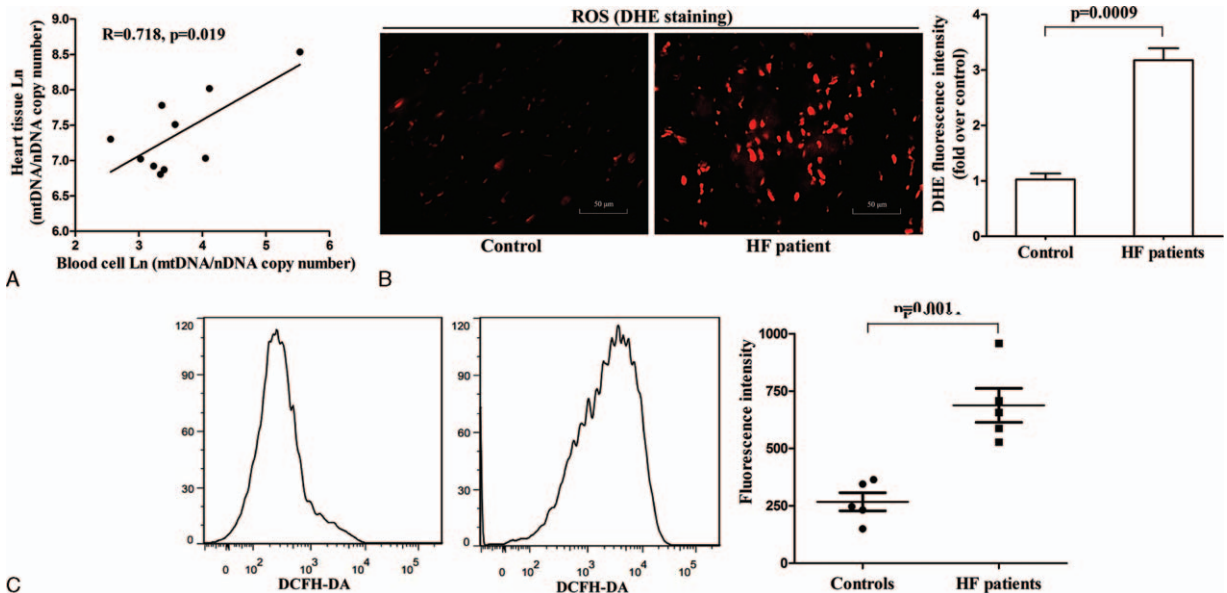

FIGURE 4. The correlation between peripheral blood cells and cardiomyocytes. (A) The correlation of mtDNA contents between peripheral leukocytes and heart tissues from end-stage heart failure patients in a subgroup of patients (n = 10) quantified by real-time quantitative PCR analysis using the nuclear gene *HBB* as an endogenous control. (B) Representative fluorescence imagines of reactive oxygen species (ROS) productions (red fluorescent) in cardiomyocytes incubated with dihydroethidium (DHE) (left panel, scale bars: 50 mm). The average levels of fluorescence intensity of DHE form control group (n = 3) and patients (n = 5) were summarized in right panel. (C) ROS generation in peripheral lymphocytes from heart failure patients or control subjects (left panel). In the right panel, Y axis represents average fluorescence intensity of DCFH-DA counted per 10,000 cells by flow cytometry (n = 5 for each group).

Strengths of our study include large population size, enrollment of HF patients with different etiologies, detailed clinical information, measurements of mtDNA copy number in triplicate with strict correction, and comprehensive analyses of the relationship of mtDNA copy number with both the risk and the prognosis of HF. However, we still have several limitations. We only tested the baseline mtDNA copy number when the patients were in hospital. Whether the mtDNA copy number would show a stable level in the process of disease progression or drug intervenes for each patient is still unknown. However, high intra-assay reliability has been confirmed by testing the same sample in different time of one day and separate days for the control subjects.[22] Although our data indicate that mtDNA content is associated with a significant increased risk for HF, the cause–effect relationship between mtDNA content and heart failure can not be fully illustrated. This is a limitation inherent in case–control study design. Prospective study based on healthy subjects and biological mechanism study will provide further evidence of how mitochondrial copy number variation affects the development and progression of heart failure.

CONCLUSION

In summary, this study presents the first evidence that decreased mtDNA copy number of peripheral leukocytes is independently associated with elevated risk of heart failure and long-term cardiovascular mortality. These results provide a noninvasive and effective biomarker for HF management and risk stratification. Therapeutic strategies targeting mitochondrial dysfunction should be investigated in future research of heart failure.

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