ORIGINAL RESEARCH

Significant Association Between Low Mitochondrial DNA Content in Peripheral Blood Leukocytes and Ischemic Stroke

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***Background*—**Cumulative evidence has shown that low mitochondrial DNA (mtDNA) content is related to elevated oxidative stress and atherosclerosis, which play important roles in ischemic stroke. The objective of this study was to explore the association between mtDNA content in peripheral blood leukocytes and ischemic stroke.

***Methods and Results*—**A total of 350 patients with first-ever ischemic stroke and 350 healthy controls were recruited in this case-control study. The mtDNA content in peripheral blood leukocytes was determined by quantitative real-time polymerase chain reaction. The levels of oxidized glutathione, reduced glutathione, and 8-hydroxy-2′-deoxyguanosine were measured by ELISA kits. Multivariate logistic regression models were used to analyze the relationship between mtDNA content in peripheral blood leukocytes and ischemic stroke. Our results show that mtDNA content of patients with ischemic stroke was notably lower compared with controls. A significant association was found between low mtDNA content and ischemic stroke. Furthermore, significant interactions were identified between low mtDNA and proven risk factors in patients with ischemic stroke. The levels of oxidized glutathione and 8-hydroxy-2′-deoxyguanosine were significantly greater in patients with ischemic stroke compared with controls.

***Conclusions*—**Our results demonstrate that low mtDNA content in peripheral blood leukocytes is associated with ischemic stroke. The relationship of low mtDNA content and ischemic stroke was particularly notable in individuals who had low mtDNA content combined with diabetes mellitus, metabolic syndrome, or cigarette smoking. Oxidative stress may be one of the contributory factors to decreased mtDNA content in patients with ischemic stroke. **(*J Am Heart Assoc*. 2017;6:e006157. DOI: 10.1161/JAHA.117.006157.)**

Key Words: association study • ischemic stroke • mitochondrial DNA content

Stroke is the third leading cause of death and an important cause of adult disability in Taiwan. The majority of stroke cases are ischemic stroke.[1] Accumulating evidence shows that oxidative stress plays a key role in the development of ischemic stroke. Mitochondria are the major cellular source of reactive oxygen species (ROS). Oxidative damage to mitochondria is likely to lead to increased production of ROS through disruption of respiratory function.[2] Excess mitochondria-derived ROS from any cause can induce endothelial dysfunction,[3] which is closely associated with the development of atherosclerosis and atherosclerotic plaque,[4] which are critical etiologic factors of ischemic stroke.

Human mitochondrial DNA (mtDNA) is 16 569-bp long and encodes 13 polypeptides of the electron transport chain and ATP synthase.[5] Changes in the biogenesis and function of mitochondria have been documented in cardiovascular disease (CVD), atherosclerosis, metabolic syndrome (MS), and diabetes mellitus (DM).[6–13] For example, Karamanlidis and coworkers[6] reported that the mtDNA content and mtDNA-encoded proteins were significantly reduced in the failing human heart, indicative

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Accompanying Data S1 and Figure S1 are available at http://jaha.ahajournals.org/content/6/11/e006157/DC1/embed/inline-supplementary-material-1.pdf

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Clinical Perspective

What Is New?

- Our study demonstrated the association between low mitochondrial DNA content in peripheral blood leukocytes and ischemic stroke.
- Synergistic effects were observed between diabetes mellitus, metabolic syndrome, cigarette smoking, and low mitochondrial DNA content on ischemic stroke risk.

What Are the Clinical Implications?

- Other than traditional cardiovascular risk factors, mitochondrial dysfunction may promote ischemic stroke risk.

of impaired mitochondrial biogenesis. They also observed a strong correlation between mtDNA content and mRNA levels of mtDNA-encoded genes. Another population-based study indicated that higher mtDNA content was associated with smaller left ventricular diastolic and systolic diameters and volumes and improved left ventricular systolic and diastolic function.[7] Chien et al found that atherogenesis was associated with decreased amounts of mtDNA among patients with DM.[8] Kim et al showed that there was a negative association between leukocyte mtDNA and MS after adjustment for potential confounding variables.[11] Significantly lower mtDNA content was observed in patients with type 2 DM compared with patients with normal glucose tolerance.[8] Recently, a case-control study by Chen et al[13] revealed that low mtDNA content in peripheral blood leukocytes was associated with an increased risk of coronary heart disease.

Although several studies have demonstrated an association between mtDNA mutations and increased risk of ischemic stroke,[10,14,15] there are few studies that have investigated the association between mtDNA content in peripheral blood leukocytes and the risk of ischemic stroke. Furthermore, cumulative evidence shows that low mtDNA content is related to elevated oxidative stress and atherosclerosis, which play important roles in ischemic stroke. Therefore, the aim of this study was to determine whether there is a relationship between mtDNA content in peripheral blood leukocytes and ischemic stroke risk using an epidemiologic study design.

Methods

Participants and Study Design

A total of 350 patients with first-ever ischemic stroke and 350 controls without stroke were recruited in this case-control study. The patients with ischemic stroke were randomly selected from the Formosa Stroke Genetic Consortium (FSGC), which enrolled nearly 1700 patients with ischemic stroke between 2005 and 2010. FSGC is a platform for hospital collaborations on studies related to the molecular biology of cerebrovascular diseases. All cases of ischemic stroke from the FSGC were confirmed with computed tomography and/or MRI. The subtypes of ischemic stroke were classified according to the TOAST (Trial of Org 10172 in Acute Stroke Treatment) criteria.[16] A tube of blood sample was collected from each patient during 3 days to 1 month post stroke. The details of the FSGC are described in a previous study.[17] Controls were recruited from those who underwent a physical examination program at Taipei Medical University Hospital and Taipei Medical University Shuang-Ho Hospital. After excluding patients with stroke history and frequency matching with cases by age (±5 years) and sex in a ratio of 1:1, a total of 350 control patients were randomly selected from 1150 healthy candidates. This study was performed in accordance with the Declaration of Helsinki and approved by the institutional review board for human subjects from Taipei Medical University. A written informed consent for blood sampling and genetic analysis was obtained from each patient before participation in this study.

Data Collection

Briefly, a structured questionnaire including demographic data, major CVD risk factors, blood biochemistry data, and medical history was collected by FSGC-trained study nurses or research assistants. Participants with a smoking habit were defined as individuals who had smoked >100 cigarettes during their lifetime. Alcohol drinking was defined as the consumption of at least one alcoholic drink per day for more than 1 year. Hypertension was defined as blood pressure (BP) >140/90 mm Hg, the presence of chronic hypertension as diagnosed by a physician, or the use of antihypertensive medication. DM was defined by a clinical diagnosis of DM, a fasting glucose level >126 mg/dL, or the taking of DM medication. Body mass index was calculated as weight in kilograms divided by the square of height in meters. According to the National Cholesterol Education Program's Adult Treatment Panel III definition for MS, a participant was deemed to have the disease when ≥3 of the following criteria were satisfied: (1) waist circumference >102 cm in men and >88 cm in women; (2) triglyceride level ≥1.7 mmol/L; (3) high-density lipoprotein cholesterol <1.0 mmol/L in men and <1.3 mmol/L in women; (4) BP ≥130/85 mm Hg or known treatment for hypertension; and (5) fasting glucose level of ≥6.1 mmol/L or known treatment for DM.

Determination of mtDNA Content in Peripheral Blood Leukocytes

Genomic DNA used for mtDNA quantification was extracted from peripheral blood leukocytes by using a nonorganic

purification method.[18] The ratio of mtDNA to nuclear DNA is often used as an estimate for the number of mtDNA per cell.[19–22] For this purpose, 2 genes, a D-loop segment of human mtDNA sequence and nuclear DNA β_2-microglobulin, were selected to quantify the mtDNA. The nuclear DNA β_2-microglobulin was used to normalize the mtDNA amount per cell. A comparative C_T method ($\Delta\Delta C_T$ method) was performed according to the Applied Biosystems protocol for relative quantitation. All samples were assayed in duplicate using the StepOne Real-Time Polymerase Chain Reaction System (Applied Biosystems) and the TaqMan method. The details of the method are described in Data S1.

Determination of Oxidative Stress and Markers Related to Mitochondrial Function in Plasma

Because of the limited funding and the available plasma specimens, two hundreds of study subjects were randomly selected from the participants with available plasma samples. Measurement of total glutathione (GSH) and oxidized GSH (GSSG) in plasma was performed by an enzymatic recycling method, using GSH reductase, according to the instructions of a GSH assay kit (Cayman Chemical). In brief, all plasma samples were deproteinated before assaying. An equal volume of 10% metaphosphoric acid was added to the sample. After centrifuge at 10 000*g* for 10 minutes, 20 μL of 4 mol/L triethanolamine reagent was added for measurement of the total GSH levels in the sample. For quantification of GSSG, 4 μL of 1 mol/L 2-vinylpyridine solution was added to the deproteinated sample. After incubating at room temperature for 60 minutes, the sample was used to measure the GSSG levels. Finally, the total GSH and GSSG concentration was then determined by the end point method according to the procedure from the assay kit. The concentration of reduced GSH was calculated as total GSH−GSSG.

Measurement of 8-hydroxy-2′-deoxyguanosine (8-OHdG) in plasma was performed by a competitive ELISA method using 8-OHdG ELISA kits from BioVision Inc and determined the levels of peroxisome proliferator-activated receptor γ coactivator 1α (PPARGC1A, also known as PGC1A) and mitochondrial uncoupling protein 2 (UCP2) in plasma were performed by a quantitative sandwich enzyme immunoassay technique using human PPARGC1A ELISA kits and human UCP2 ELISA kits from CUSABIO Biotech following manufacturer's protocol. Briefly, 8-OHdG, PPARGC1A, and UCP2 were detected using a fluorescence microplate reader at 450 nm. For PPARGC1A and UCP2, wavelength correction was performed at subtract readings at 540 nm from the readings at 450 nm. The amounts of 8-OHdG, PPARGC1A, and UCP2 were calculated using a standard curve generated from the standard solution.

Statistical Analysis

Based on power calculations (assuming equal group size) for logistic regression, 266 to 776 study patients were needed to provide 80% power to detect a statistically significant (α=0.05) odds ratio (OR) of 2.0~1.5. Student *t* test was used to compare continuous variables between groups in Table 1. In the same table, chi-square test was applied to test differences in categorical variables. In Table 2, univariate and multivariate logistic regression models were used to evaluate the association of mtDNA content with ischemic stroke. Age, sex, smoking, and alcohol drinking–corrected Spearman rank partial correlation coefficients were calculated to evaluate the relationship between mtDNA and clinical data in Table 3. A Kolmogorov–Smirnov test was used to check normality. Because the distribution of mtDNA

Table 1. Characteristics of Ischemic Stroke Cases and Controls

Variables	Cases n=350	Controls n=350	*P* Value
Age, mean±SD, y	60.9±9.1	60.4±9.1	0.483
Sex, No. (%)			
Male	246 (70.3)	246 (70.3)	1.000
Female	104 (29.7)	104 (29.7)	
Smoking, No. (%)			
Yes	181 (51.7)	111 (31.7)	<0.001
No	169 (48.3)	239 (68.3)	
Alcohol drinking, No. (%)			
Yes	74 (21.1)	44 (12.6)	0.003
No	276 (78.9)	306 (87.4)	
MS, No. (%)			
Yes	230 (65.7)	140 (40.0)	<0.001
No	120 (34.3)	210 (60.0)	
Hypertension, No. (%)			
Yes	252 (72.0)	184 (52.6)	<0.001
No	98 (28.0)	166 (47.4)	
DM, No. (%)			
Yes	160 (45.7)	61 (17.4)	<0.001
No	190 (54.3)	289 (82.6)	
Dyslipidemia, No. (%)			
Yes	288 (82.3)	277 (79.1)	0.292
No	62 (17.7)	73 (20.9)	
BMI, mean±SD, kg/m^2	25.5±3.7	24.7±3.0	0.001

Student *t* test was used for continues variables and chi-square test was used for categories variables. There were no missing data for all variables. BMI indicates body mass index; DM, diabetes mellitus; MS, metabolic syndrome.

Table 2. Traditional Risk Factors, mtDNA Content, and Risk of Ischemic Stroke

Variables	Group	OR (95% CI)	aOR* (95% CI)
Smoking	No	1.0	1.0
	Yes	2.31 (1.70–3.14)	2.34 (1.50–3.65)
Alcohol drinking	No	1.0	1.0
	Yes	1.87 (1.24–2.80)	1.68 (0.97–2.90)
MS	No	1.0	1.0
	Yes	2.88 (2.11–3.91)	1.68 (1.08–2.63)
Hypertension	No	1.0	1.0
	Yes	2.32 (1.70–3.18)	1.70 (1.11–2.60)
DM	No	1.0	1.0
	Yes	3.99 (2.82–5.65)	3.08 (1.96–4.83)
mtDNA content†	≥1.09	1.0	1.0
	<1.09	11.77 (8.26–16.77)	11.73 (7.91–17.39)

aOR indicates adjusted odds ratio; CI, confidence interval; OR, odds ratio.
*Adjusted for age, sex, body mass index, hypertension, diabetes mellitus (DM), metabolic syndrome (MS), mitochondrial DNA (mtDNA) content, smoking, and alcohol drinking as appropriate.
†The median value of mtDNA content from all study patients was used as the cut point.

content and oxidative stress markers did not meet the normality assumption, a Wilcoxon rank sum test and generalized linear models adjusting for age, sex, DM, hypertension, smoking, alcohol drinking, and body mass index were used in Table 4. A Wilcoxon rank sum test and a Kruskal–Wallis test were used to compare medians in Figure 1 and Figure S1, respectively. A Rothman's synergy index[23] (S index) was used to measure the interaction on an additive scale in Figure 2. The confidence interval (CI) of the S index was estimated with the method described by Hosmer and Lemeshow.[24] A synergistic interaction was defined as an S index >1. Spearman rank partial correlation coefficients with age, sex, smoking, and alcohol drinking-adjusted were used to determine the correlations of mtDNA content with the markers related to mitochondrial function in Figure 3. *P* values for 2-tailed tests were calculated, and statistical significance was set at $P<0.05$. Analyses were performed using SAS statistical software 9.1 (SAS Institute).

Table 3. Correlations Between mtDNA Content and Clinical Data in All Study Patients

Variables	Spearman Partial Correlation Coefficients*	*P* Value
Age	−0.14	<0.005
Male	0.02	0.633
Smoking	−0.15	<0.0001
Alcohol drinking	−0.04	0.260
SBP	−0.36	<0.0001
DBP	−0.19	<0.0001
Fasting glucose	−0.13	<0.005
Total cholesterol	−0.01	0.759
HDL-C	0.08	0.027
LDL-C	−0.02	0.577
Triglyceride	−0.16	<0.0001
BMI	−0.04	0.328
Waist-hip ratio	−0.19	<0.0001
MS	−0.18	<0.0001

BMI indicates body mass index; DBP, diastolic blood pressure; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; MS, metabolic syndrome; SBP, systolic blood pressure.
*Adjusted for age, sex, smoking, and alcohol drinking as appropriate.

Results

Characteristics of Study Participants

A total of 700 study patients including 350 patients with ischemic stroke and 350 controls were recruited in this study. The basic characteristics of patients with ischemic stroke and controls are shown in Table 1. The distribution of cigarette smoking, alcohol drinking, MS, hypertension, and DM were significantly different between patients and controls. These risk factors were more common in patients with ischemic stroke. Dyslipidemia and overweight were also more frequently observed in patients with ischemic stroke, but the difference did not reach statistical significance.

Association of mtDNA Content in Peripheral Blood Leukocytes With Ischemic Stroke

As shown in Figure 1, the median of mtDNA content from peripheral blood leukocytes in patients with ischemic stroke was significantly lower compared with controls. The relationships between traditional risk factors, mtDNA content, and ischemic stroke are shown in Table 2. A significantly higher risk of ischemic stroke was found in patients with cigarette smoking (OR, 2.31; 95% CI, 1.70–3.14), alcohol drinking (OR, 1.87; 95% CI, 1.24–2.80), MS (OR, 2.88; 95% CI, 2.11–3.91), hypertension (OR, 2.32; 95% CI, 1.70–3.18), and DM (OR, 3.99; 95% CI, 2.82–5.65). There was also a strong association between the lower mtDNA content and ischemic stroke (OR, 11.77; 95% CI, 8.26–16.77). The results remained mainly unchanged in a multivariate logistic regression model (OR, 11.73) that adjusted for age, sex, hypertension, DM, MS, body mass index, smoking, and alcohol drinking. We also compared the mtDNA content in different subtypes of ischemic stroke, and no significant difference was observed according to Kruskal–Wallis test ($P=0.446$) (Figure 1).

Table 4. Oxidative Stress Markers and mtDNA Content in Patients With Ischemic Stroke and Controls

Variables	Unit	Cases (n=150)	Controls (n=50)	*P* Value*	Adjusted *P* Value†
GSSG‡	μmol/L	1.83 (1.09–3.01)	0.79 (0.35–1.44)	<0.0001	0.011
GSH/GSSG‡	…	1.72 (1.59–1.83)	1.80 (1.66–1.85)	0.219	0.356
8-OHdG‡	pg/mL	6.33 (4.33–10.19)	4.87 (3.92–5.76)	0.001	0.076
mtDNA content‡	…	0.90 (0.78–0.99)	1.20 (1.04–1.36)	<0.0001	<0.0001

GSH indicates glutathione; GSSG, oxidized glutathione; mtDNA, mitochondrial DNA; 8-OHdG, 8-hydroxy-2′-deoxyguanosine.
*Wilcoxon rank sum tests were used to compare medians between cases and controls.
†Adjusted *P* value was calculated by generalized linear models adjusting for age, sex, diabetes mellitus, hypertension, smoking, alcohol drinking, and body mass index.
‡Median (quartile 1–quartile 3).

Effect of mtDNA Content With Traditional Risk Factors of CVD on Ischemic Stroke

In order to further analyze the interaction between traditional risk factors and mtDNA content, 5 common traditional CVD risk factors (MS, hypertension, DM, smoking, and alcohol drinking) were selected to investigate their impacts with mtDNA on ischemic stroke. Study patients were stratified into 4 groups according to risk factor status and level of mtDNA content (Figure 2). Compared with the patients with high mtDNA content and without MS, the proportion of patients with ischemic stroke was increased in patients with only low mtDNA content or only MS, and the proportion of patients with ischemic stroke was further increased in patients with both low mtDNA content and MS (S index=2.52; 95% CI, 1.47–4.31). Similar interaction effects were observed between low mtDNA content and smoking (S index=2.07; 95% CI, 1.17–3.67) and low mtDNA content and DM (S index=3.50; 95% CI, 1.83–6.72). However, there were no synergistic effects between hypertension, alcohol drinking, and mtDNA content on ischemic stroke.

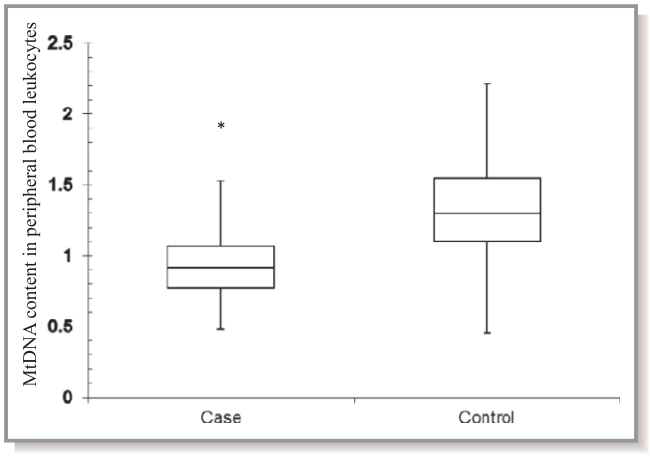

Figure 1. Box plot analysis illustrating the distribution of peripheral blood leukocytes mitochondrial DNA (mtDNA) content in patients with ischemic stroke and controls. The mtDNA content (the amount of mtDNA normalized to nuclear gene and relative to a calibrator DNA) is shown on the *y* axis. The peripheral blood leukocytes mtDNA content of patients with ischemic stroke was significantly lower compared with controls according to Wilcoxon rank sum test (*P<0.0001). Horizontal lines: group medians; boxes: 25% to 75% quartiles, range, peak, and minimum.

Correlations Between mtDNA Content and Clinical Data

In Spearman partial correlation analysis (Table 3), our data indicated that peripheral blood leukocytes mtDNA content was negatively correlated with age (ρ=−0.14), smoking (ρ=−0.15), systolic BP (ρ=−0.36), diastolic BP (ρ=−0.19), fasting glucose (ρ=−0.13), triglycerides (ρ=−0.16), and waist-hip ratio (ρ=−0.19) after being adjusted for sex, age, smoking, and alcohol drinking. In contrast, peripheral blood mtDNA content was positively correlated with high-density lipoprotein cholesterol (ρ=0.08).

Oxidative Stress Marker and mtDNA Content in Patients With Ischemic Stroke and Controls

The levels of GSSG, GSH/GSSG ratio, and 8-OHdG in plasma were used to determine oxidative stress. As shown in Table 4, the medians of GSSG and 8-OHdG were significantly greater in patients with ischemic stroke compared with controls. After adjusting for age, sex, DM, hypertension, smoking, alcohol drinking, and body mass index, the concentration of GSSG was still significantly greater in patients with ischemic stroke and the greater level of 8-OHdG in patients with ischemic stroke turned to borderline significance. The results indicated that oxidative stress was significantly greater in patients with ischemic stroke compared with controls. However, mtDNA content was lower in patients with ischemic stroke compared with controls.

Association of mtDNA Content With Markers Related to Mitochondrial Function

To examine whether reduced mtDNA content indicated mitochondrial dysfunction, we measured the levels of

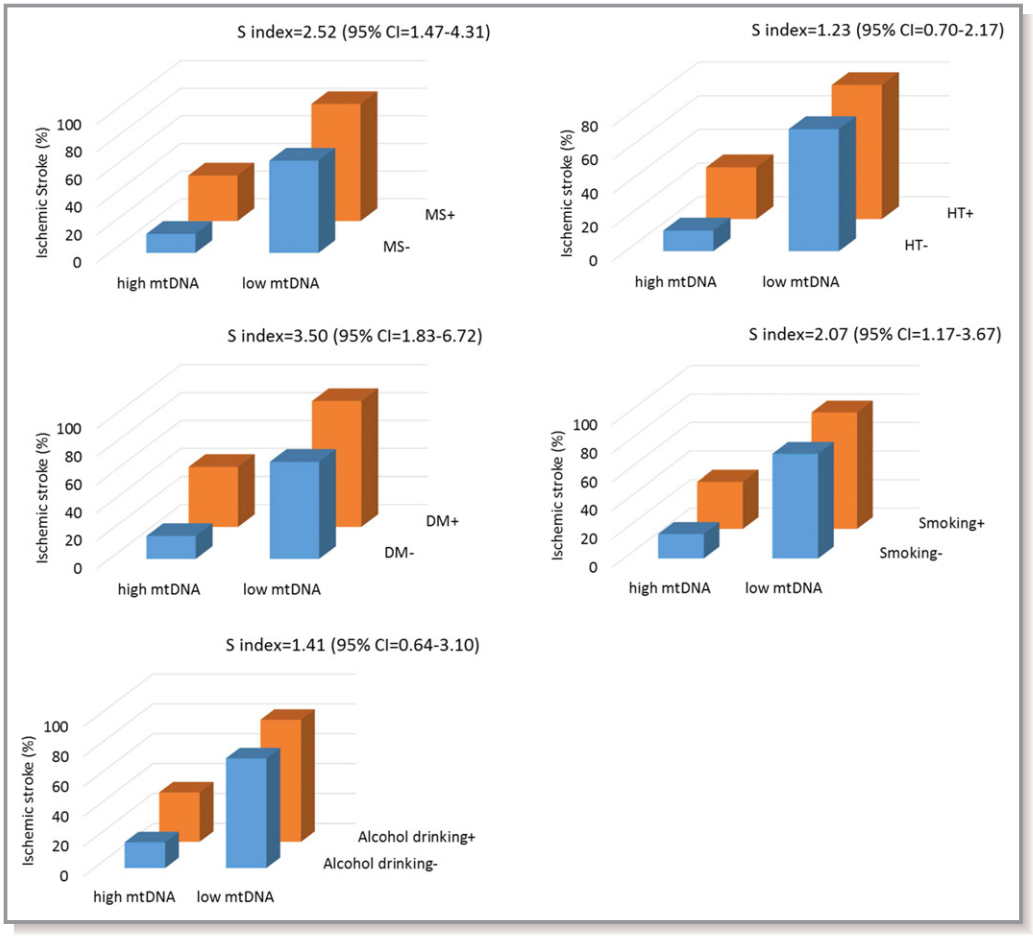

Figure 2. Synergistic effects between mitochondrial DNA (mtDNA) content and traditional risk factors of cardiovascular disease on ischemic stroke. High mtDNA was defined as mtDNA content ≥1.09 and low mtDNA was defined as mtDNA content <1.09. The proportion of patients with ischemic stroke in different combinations of risk factors is shown on the *y* axis. A synergistic interaction was defined as an S index >1. Synergistic effects were observed between low mtDNA content and smoking (S index=2.07; 95% confidence interval [CI], 1.17–3.67); low mtDNA content and diabetes mellitus (DM; S index=3.50; 95% CI, 1.83–6.72); and low mtDNA content and metabolic syndrome (MS; S index=2.52; 95% CI, 1.47–4.31). HT indicates hypertension.

PPARGC1A and UCP2, which involved mitochondrial biogenesis and mediated proton leak across the inner membrane of mitochondria as the indicators of mitochondria function. In Figure 3A, mtDNA content ≥1.09 was classified as high mtDNA content and <1.09 as low mtDNA content. Both PPARGC1A and UCP2 were negatively correlated with high mtDNA content. In Figure 3B, a significantly moderate negative correlation was found between mtDNA content (continuing value) and UCP2 ($\rho=-0.31$, $P=0.005$) after adjusting for age, sex, smoking, and alcohol drinking.

Discussion

This is the first molecular epidemiologic study to demonstrate the negative association between peripheral blood mtDNA content and ischemic stroke. Previously, most neurologists focused on the role of mitochondria in mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke syndrome, which are progressive neurodegenerative disorders.[25] Only a few studies investigated the relationship between mtDNA mutations and the risk of ischemic stroke either using a genome-wide association study or a haplogroup association study.[10,14,15] In the present study, we found that patients with ischemic stroke exhibited strikingly lower peripheral blood mtDNA content compared with healthy controls. Similar results have been reported from studies of CVDs. Chen et al observed that low mtDNA content in peripheral blood leukocytes was linked to an increased risk of coronary heart disease.[13] Ide et al[26] reported that the mtDNA content was preferentially decreased by 44% in a murine model of

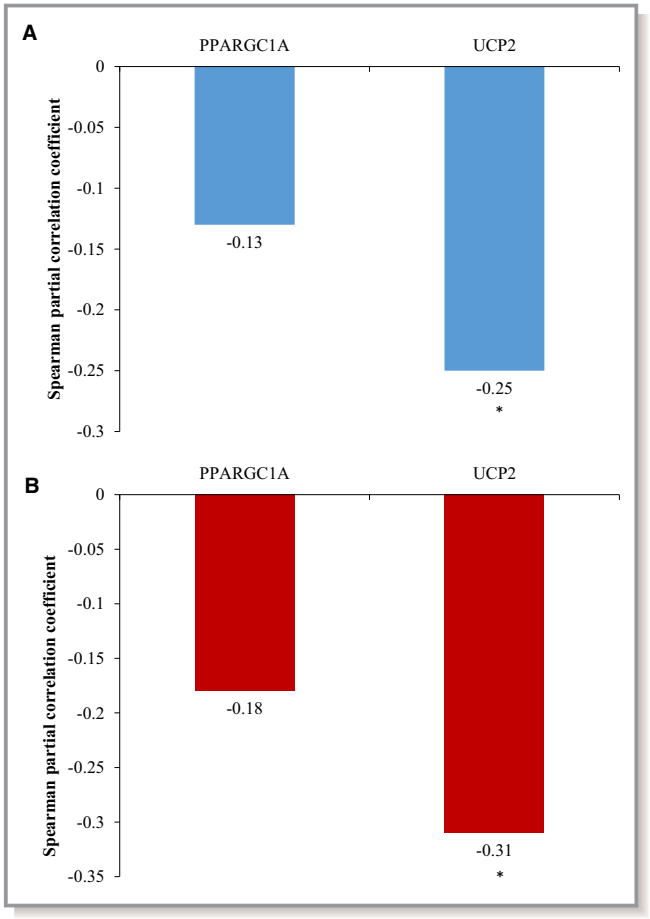

Figure 3. Correlations of mitochondrial DNA (mtDNA) content with levels of PPARGC1A and UCP2 were determined by Spearman rank partial correlation coefficient analyses after adjusting for age, sex, smoking, and alcohol drinking. A, Blue bars show Spearman partial correlation coefficients (ρ) for high mtDNA content (cutoff point ≥1.09) vs peroxisome proliferator-activated receptor γ coactivator 1α (PPARGC1A) and uncoupling protein 2 (UCP2). B, Red bars show partial correlation coefficients (ρ) for mtDNA content (continuing value) vs PPARGC1A and UCP2. A significantly moderate negative correlation was found between mtDNA content and UCP2. Although PPARGC1A also negatively correlated with mtDNA content, the result did not reach statistical significance. The asterisk indicates a statistically significant correlation with $P<0.05$.

myocardial infarction. In addition, previous studies also demonstrated that decline of leukocyte mtDNA content was involved in atherogenesis,[27,28] which played an important role in the development of ischemic stroke.[29,30] A recent study reported a significantly positive correlation between mtDNA content in peripheral blood mononuclear cells and mtDNA content in atherosclerotic plaque tissue.[13] This finding indicates that leukocyte mtDNA content could be used as a surrogate of atherosclerotic plaque mtDNA content. Notably, measuring mtDNA content from peripheral blood samples is more acceptable to most patients. In our study, no significant difference was observed for mtDNA content in different subtypes of ischemic stroke. Although the mechanisms for different types of ischemic stroke may be different, atherosclerosis may account for the majority of these ischemic stroke subtypes. For patients with cardioembolism, many had atrial fibrillation and coronary artery disease. Atrial fibrillation is often considered a prothrombotic state. Therefore, the mtDNA level in different types of ischemic stroke may be similar.

Mitochondrial dysfunction can be caused by impaired mitochondrial biogenesis, which can be revealed by a decrease of mtDNA content. Increased oxidative stress may contribute to alterations in the copy number and integrity of mtDNA in human cells in pathological conditions.[31,32] Decreased mtDNA content was reported to result in excess mitochondrial ROS production.[33] Increased production of ROS, in turn, leads to oxidative damage to lipids, proteins, and mtDNA.[34] Furthermore, mtDNA damage may lead to decreased expression of constituent polypeptides of respiratory enzymes or assembly of defective respiratory enzymes that produce more ROS.[35] ROS-induced ROS release may amplify and target ROS signals under physiological conditions; however, when excessive, may contribute to the pathogenesis of endothelial dysfunction, atherosclerosis,[36,37] and the evolution of CVD.[38] Consistent with these basic research findings, our results showed that patients with ischemic stroke exhibited significantly lower mtDNA content compared with controls, suggesting a marked relationship between low mtDNA content in peripheral blood leukocytes and ischemic stroke.

The results of our study demonstrated interaction effects between MS, DM, smoking, and mtDNA content, in the modulation of ischemic stroke risk. Previously, several studies have reported high oxidative stress levels in patients with MS,[39,40] patients with DM,[41,42] and in smokers.[43,44] We also found significantly negative correlations between mtDNA content with BP, blood glucose, triglyceride, and waist-hip ratio, which were in line with findings from previous studies.[45,46] Increased ROS was a common feature in MS, DM, smoking, and mitochondrial dysfunction. Cumulative oxidative stress may eventually accelerate ischemic stroke process. The underlying mechanisms of interaction effect between mtDNA and host variables on the risk of ischemic stroke need further study.

In our study, we found greater oxidative stress in patients with ischemic stroke compared with controls. However, mtDNA content was lower in patients with ischemic stroke compared with controls. One possible explanation may be that oxidative damage to mtDNA polymerase gamma declined mtDNA replicaiton and eventually led to loss of mitochondrial function.[18] Oxidative stress may be one of the contributory factors to decreased mtDNA content in patients with ischemic stroke. We also found a significantly moderate negative correlation between mtDNA content and UCP2 and a weak

negative correlation with PPARGC1A. Previous studies indicate that mitochondrial dysfunction induced by oxidative stress may lead to UCP2 and PPARGC1A expression.[47,48] Therefore, according to our results, we speculate that the decreased mtDNA is associated with mitochondrial dysfunction.

Study Limitations

There are several potential limitations in this study. First, a case-control study design was employed and mtDNA content was measured after disease onset. Thus, a reverse causality on the correlation between mtDNA content and ischemic stroke is possible. Previous reports indicate that mtDNA content appear to have high heritability[49] and negative correlations with increased ROS, endothelial cells dysfunction, and atherosclerosis. All current knowledge of mtDNA supports the view that low mtDNA content is more likely to be a risk factor for ischemic stroke. Second, only Han Chinese patients were selected in this study. It is necessary to confirm this association in other populations. Third, there was a lack of a direct connection between changes in peripheral blood leukocytes mtDNA and incidence of stroke. Further research using a cohort study design is needed to clarify the relationship between reduced mtDNA and incidence of stroke. Finally, our study was observational in design, thus we cannot rule out the presence of unknown confounding variables, not accounted for in the final analysis.

Conclusions

Low mtDNA content in peripheral blood leukocytes was significantly correlated with ischemic stroke. Synergistic effects were observed between MS, DM, smoking, and low mtDNA content on ischemic stroke risk. Oxidative stress may be one of the contributing factors to decreased mtDNA content in patients with ischemic stroke. Further studies are warranted to explore the underlying mechanism of the effect of mtDNA content on ischemic stroke. Stroke is among the most frequent causes of death and adult disability in highly developed countries. However, treatment options remain limited. Mitochondria-targeted antioxidants may be a future option for treatment of ischemic stroke.

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Disclosures

None.

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SUPPLEMENTAL MATERIAL

Data S1.

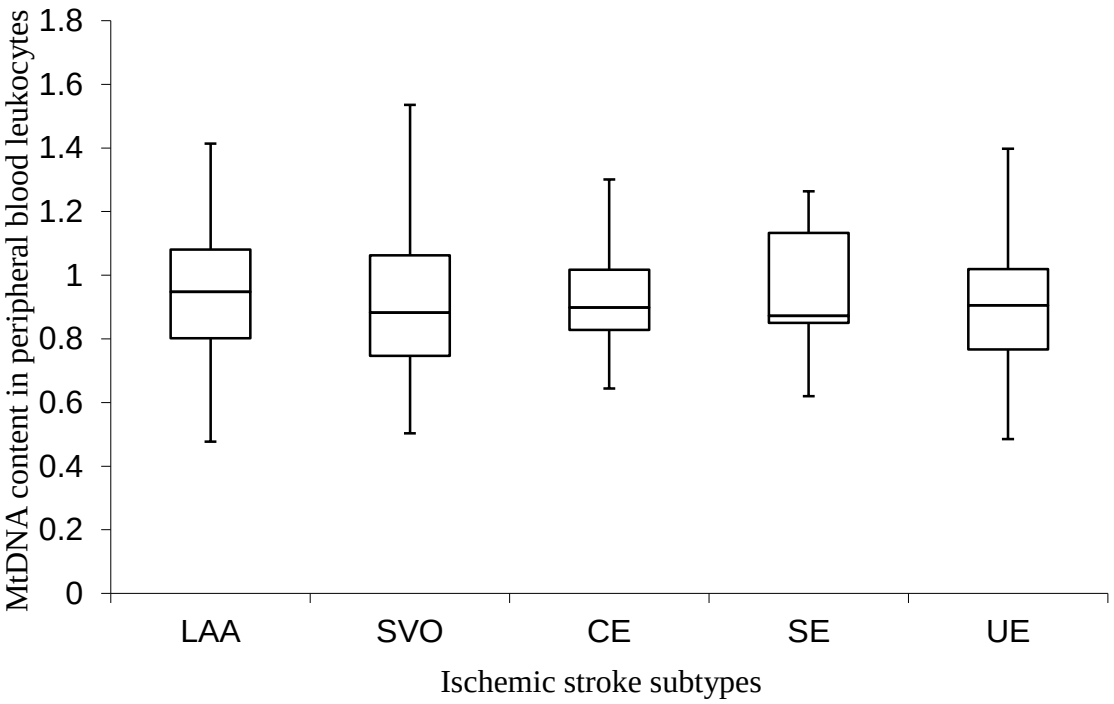
Supplemental Methods

Determination of mtDNA content in peripheral blood leukocytes

Genomic DNA used for mtDNA quantification was extracted from peripheral blood leukocytes by using a non-organic purification method.[1] The ratio of mtDNA to nuclear DNA is often used as an estimate for the number of mtDNA per cell.[2-5] For this purpose, two genes, a D-loop segment of human mtDNA sequence and nuclear DNA β-2-microglobulin (β2M), were selected to quantify the mtDNA. The nuclear DNA β2M was used to normalize the mtDNA amount per cell. A comparative C_T method ($\Delta\Delta C_T$ method) was performed according to the Applied Biosystems protocol for relative quantitation. All samples were assayed in duplicate using the StepOneTM Real-Time PCR System (Applied Biosystems, USA) and the TaqMan method. A calibrator DNA (the same DNA sample for all runs) and a negative control without a DNA template were included in each run. The thermal cycling conditions consisted of 50℃ for 2 min followed by an initial denaturation step at 95℃ for 10 min, 40 cycles at 95℃ for 15 s and 60℃ for 1 min. The quantitative real-time polymerase chain reactions (qRT-PCRs) for mtDNA were performed in a 20-μL solution containing 2× *Taq*Man Universal Master Mix II with UNG (Applied Biosystems, USA), 20× MT-7S *Taq*Man assay (Hs02596861_s1, Applied Biosystems, USA) and 20 ng of template DNA. The sequences of the primers and probe used in the qRT-PCR reaction for β2M were as follows: 5'-GCCCGCTAAGTTCGCATGT-3' (forward primer), 5'-TTCGAAACCGCTTTGTATCACA-3' (reverse primer) and 5'FAM-CACGCGACGTTTGT-3'MGB (probe) (Applied Biosystems, USA). The corresponding RT-PCR efficiencies (E) of one cycle in the exponential phase were

calculated according to the equation: $E=10^{-1/slope}-1$. Investigated genes showed high RT-PCR efficiency levels for D-loop ($E_{D\text{-}loop}$=1.013) and β2M ($E_{\beta 2M}$=1.021) in the investigated range from 0.31 to 40 ng of DNA input (n=8, 2-fold serial dilutions) with high linearity (Pearson correlation coefficient r>0.99). Because $E_{D\text{-}loop}$ and $E_{\beta 2M}$ were approximately equal and close to 1, the mtDNA content was determined by the $2^{-\Delta\Delta CT}$ method as described by Livak and Schmittgen,[6] where $\Delta\Delta C_T$= (D-loop C_T – β2M $C_T)_{sample}$ – (D-loop C_T – β2M $C_T)_{calibrator}$, and which meant that the amount of mtDNA(D-loop) was firstly normalized to nuclear DNA(β2M) in each sample and then relative to the calibrator in order to standardize the data in different runs.

Figure S1. Box plot analysis illustrating the distribution of mtDNA content in peripheral blood leukocytes among patients with different ischemic stroke subtypes.



The mtDNA content is shown on the Y axis. Different ischemic stroke subtypes are shown on the X axis. No significant difference was observed among peripheral blood leukocytes mtDNA content and ischemic stroke subtypes according to the Kruskal-Wallis test (P=0.446). Horizontal lines: group medians; boxes: 25-75% quartiles, range, peak and minimum. Abbreviations: LAA, large-artery atherosclerosis, SVO, small vessel occlusion, CE, cardioembolism, SE, stroke of specific etiology, UE, stroke of undetermined etiology.

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