

RESEARCH ARTICLE

Detection of mitochondrial DNA with 4977 bp deletion in leukocytes of patients with ischemic stroke

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Abstract

Background

Coronary artery disease is associated with a common mitochondrial DNA alteration, a 4977 bp deletion (mtDNA⁴⁹⁷⁷). The role of mtDNA⁴⁹⁷⁷ in ischemic stroke is unknown.

Methods

Real-time quantitative PCR was performed to quantify total mtDNA and mtDNA⁴⁹⁷⁷ in leukocytes in 283 ischemic stroke cases and 135 controls. Ratios of mtDNA⁴⁹⁷⁷ to total-mtDNA and total-mtDNA to nuclear-DNA were calculated. Nested PCR and Sanger sequencing were used to confirm undetectable levels of mtDNA⁴⁹⁷⁷.

Results

For 191 patients and 74 control subjects in the male group and 92 patients and 61 control subjects in the female group, there were no significant between-group differences in age, cholesterol level, body mass index, stroke severity, or 4977 deletion. After adjusting for confounding factors, there was no correlation between mtDNA⁴⁹⁷⁷ amount and infarction risk, recurrent stroke, or stroke severity. However, mtDNA⁴⁹⁷⁷ was undetected in 6.94% subjects, and these individuals had a higher prevalence of stroke than those with detectable mtDNA⁴⁹⁷⁷ (OR: 0.181, 95% CI 0.041–0.798, $p = 0.024$). Additionally, mtDNA⁴⁹⁷⁷ status had no effect on stroke prognosis, including stroke severity and recurrent stroke.

Conclusion

In conclusion, there was no apparent association between mtDNA⁴⁹⁷⁷ deletion and cerebral infarction. Undetectable mtDNA⁴⁹⁷⁷ may be a marker or risk factor for ischemic stroke.

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Introduction

Mounting evidence suggests that mitochondria dysfunction and the accumulation of mitochondria DNA (mtDNA) damage plays an important role in the development of atherosclerotic lesions. MtDNA damage not only correlates with the severity of atherosclerotic lesions but also precedes initial pathological processes [1–5]. The exact mechanism of this relationship is not well understood, but may be related to the central role of mitochondria in cellular energy homeostasis [5].

Evidence also suggests that somatic mtDNA alternations are associated with coronary artery disease (CAD) and atherosclerosis. Ballinger et al. [6] showed that atherosclerotic aortas had increased numbers of mtDNA oxidative lesions compared to normal aortas. Previous studies have shown that patients with CAD have an increased abundance of mtDNA 4977 bp deletions (mtDNA⁴⁹⁷⁷) in the heart and in circulating leukocytes [7, 8]. A significantly higher prevalence of mtDNA⁴⁹⁷⁷ and higher relative amounts of the deletion were identified in CAD patients compared to healthy control subjects (26.2% versus 4.5%; $p = 0.03$ and $0.089 \pm 0.02\%$ versus $0.009 \pm 0.009\%$; $p = 0.02$) [9]. The accumulation of mtDNA⁴⁹⁷⁷ has also been implicated in human aging [10]; in one study, the amount of mtDNA⁴⁹⁷⁷ was positively associated with age, independent of traditional risk factors and clinical parameters [11].

Stroke is the third leading cause of death worldwide and is associated with a 30-day disability rate of 61.2% in Taiwan [12]. Atherothrombotic stroke, which is diagnosed when an ischemic lesion > 1.5 cm is identified on brain imaging and when there is arterial stenosis $> 50\%$ of any carotid/cerebral artery, accounts for 20% of all stroke events [13]. Atherothrombotic stroke and CAD share the same conventional risk factors, susceptibility genes, and pathophysiology [13]. Additionally, several major risk factors for ischemic stroke are associated with mitochondrial dysfunction such as that occurring in metabolic syndrome, cigarette smoke exposure, and hypercholesterolemia [14–16].

Based on the earlier work mentioned in the Introduction section above, we hypothesized that, similar to coronary artery disease, stroke is associated with a mtDNA⁴⁹⁷⁷ deletion.

Recently, a genome-wide study reported an association between mitochondrial respiratory chain complex I/IV dysfunction and ischemic stroke, especially for small vessel stroke [2]. The common deletion mtDNA⁴⁹⁷⁷ occurred between nucleotides 8470 and 13459, in which five genes for tRNA and seven genes for components of complex I (ND3, ND4, ND4L, and a part of NAD5), IV (COXIII), and V of the respiratory chain (ATPase 6 and a part of ATPase 8) were compromised [4]. Mitochondrial dysfunction, particularly involving complexes I and IV, has been associated with cardiovascular disease [4], stroke [3, 8], Alzheimer's disease [17], Parkinson's disease [17–20], and psychiatric disorders [21]. While it is controversial how functions are altered by mtDNA⁴⁹⁷⁷ deletion, electron movement in the respiratory chain of mtDNA⁴⁹⁷⁷ cells is still likely to lead to reactive oxygen species (ROS) generation. Damaged mtDNA leads to the dysfunction of integral membrane protein complexes of the respiratory chain, which consequently leads to apoptotic death and cellular necrosis [3–5, 15]. Therefore, we hypothesized that the pathology of mitochondrial damage in atherogenesis may be related to alterations in the generation of reactive oxygen species and in ATP synthesis.

In the present study, we thus hypothesized that mtDNA⁴⁹⁷⁷ might play an important role in ischemic stroke. To date, little research has examined the presence of mtDNA⁴⁹⁷⁷ deletions in the cells and tissues of patients with stroke. Therefore, we evaluated mtDNA⁴⁹⁷⁷ in a cohort of patients with stroke and examined relevant associations.

Materials and methods

Patients and control subjects

Study subjects were enrolled from Chang Gung Memorial Hospital. Each patient or her/his legally acceptable representative was informed of the aim of the study. This study has been cleared by Chang Gung Memorial Hospital Institution Ethics Review Board for human studies and patients or guardians provided written informed consent prior to study participation. Ischemic stroke and its subtypes were diagnosed and classified by two neurologists based on clinical presentation and the available brain imaging data. This study enrolled patients with atherothrombotic infarction or lacunar infarction. Subtypes of cerebral infarction were defined by the TOAST criteria with modifications as follows: (1) atherothrombotic infarction was diagnosed when there was an ischemic lesion > 1.5 cm on brain imaging and arterial stenosis > 50% of any carotid/cerebral artery; (2) lacunar infarction was diagnosed when the brainstem or subcortical hemispheric ischemia was < 1.5 cm in diameter on brain imaging, and when there was no clinical evidence of cerebral cortical or cerebellar dysfunction [22]. Patients with recent stroke (< 6 months) or a medical condition such as atrial fibrillation, acute coronary syndrome, chronic renal disease, infection or inflammatory disease, other neurodegenerative disease, or cardioembolism/undetermined stroke type were excluded. Healthy control subjects were recruited randomly from the community and had no history of stroke (including hemorrhagic or ischemic stroke), neurological disease, or overt medical disease such as chronic renal failure, myocardial infarction, atrial fibrillation, and cancer.

Evaluation of mtDNA⁴⁹⁷⁷

Blood samples were collected and immediately (within 2 hours) processed for leukocyte isolation. Samples were then frozen at -80°C and stored until analysis. Leukocyte DNA was extracted using a DNA Extraction Kit according to the manufacturer's specifications (Agilent Technologies, La Jolla, CA). Nested PCR was performed to detect the presence of mtDNA⁴⁹⁷⁷ [23]. The two pairs of nested primers used for the detection of mtDNA⁴⁹⁷⁷ are shown in Fig 1. The PCR condition was set as Table 1. The PCR condition was set as pre-denaturation at 94°C for 5 min, 30 cycles at 94°C for 10 secs, 58°C for 45 secs and 72°C for 50 secs, and a final extension at 72°C for 10 min. We used the ratio of the absorbance at 260 and 280 nm (A₂₆₀/A₂₈₀) to assess the purity of nucleic acids. The DNA samples were all with good purity (260/280 = 1.7–2.0). Then we run the DNA samples by standard protocol for performing 1% agarose gel electrophoresis to detect other possible contaminants free RNA or faint smear. The samples were run in triplicates. Products were sequenced by Sanger sequencing. The presence of mtDNA⁴⁹⁷⁷ was indicated by the appearance of a 358-bp band and verified by sequencing analysis.

Quantification of mtDNA⁴⁹⁷⁷

To quantify the relative ratio of mtDNA⁴⁹⁷⁷ to total mitochondria DNA, real-time quantitative PCR (RTQ-PCR) was performed to quantify total mtDNA and mtDNA⁴⁹⁷⁷ molecules in leukocytes. TaqMan[®] Gene Expression Assays and the Sequence Detection system (ABI Prism 7900, Applied Biosystems) were used for quantification (Table 2). β -actin gene expression was measured as an indicator of nuclear DNA concentration. The common mtDNA deletion of 4,977 bp had breaking points between nucleotides 8470 and 13347, with two direct 13-bp repeats. The ND1 gene used for reference was located at nucleotides 3312 to 4122 on mtDNA and provides one of the seven mitochondrial encoded subunits of NADH dehydrogenase.

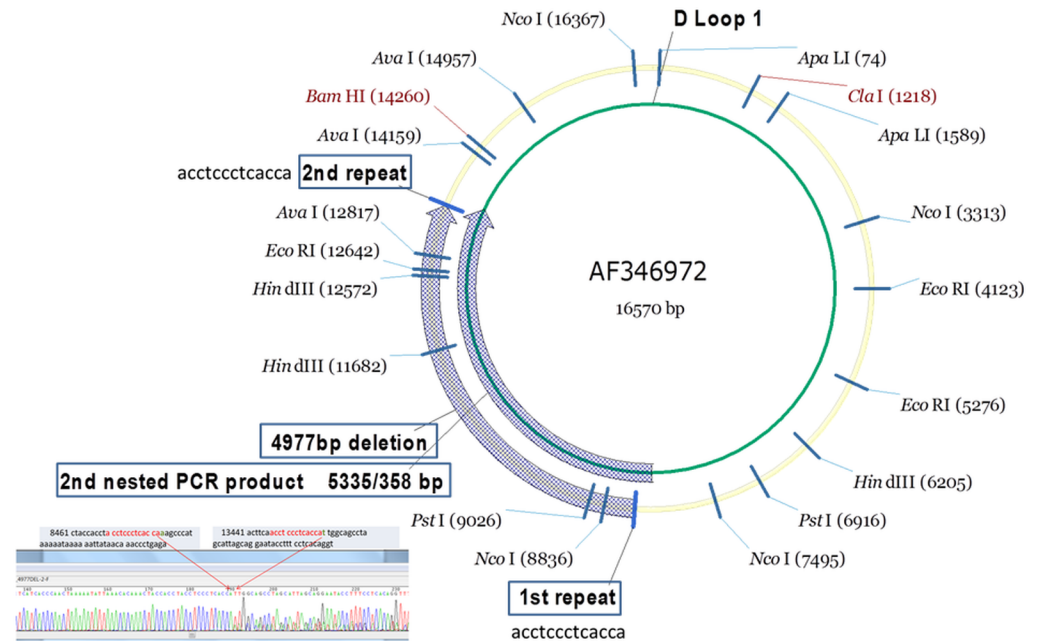


Fig 1. Detection of mtDNA⁴⁹⁷⁷ and quantification of total and deleted mtDNA in leukocytes using real-time quantitative PCR.

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Table 1. Primers using sequencing for nested PCR.

Primer	sequence (5'→3')	Tm(°C)	PCR product size(bp)
4977del-1-F	AACCACAGTTCATGCCCATC	62.1	5472/495
4977del-1-R	TGTTAGTAAGGGTGGGGAAGC	60.4	5472/495
4977del-2-F	ACCCATAGCACCCCTCTAC	58.5	5335/358
4977del-2-R	CTTGTCAGGGAGGTAGCGATG	62.1	5335/358

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Primers and TaqMan probes ordered with Applied Biosystems™ FAM™ and VIC™ dyes, used to amplify mtDNA⁴⁹⁷⁷, ND1 gene and β-actin gene. The Ct method (ΔCt) was used to calculate relative levels of mtDNA⁴⁹⁷⁷ between patients and control subjects. First, we normalized the Ct endogenous control: ΔCt sample = Ct Target (4977 deletion)—Ct Endogenous Control

Table 2. Primers and TaqMan probes for real-time quantitative PCR.

	Assay ID	Primers and VIC-probe
		Forward Primer:
		5' - GCCCACCATAATTACCCCCATAC-3'
		Reverse Primer:
		5' -GAGTAGAAACCTGTGAGGAAAGGT-3'
		Probe: FAM-CCTCATCACCCCACTAAAA-NFQ
		(Product size: 5117/140 bp)
MT-ND1	Hs02596873_s1	VIC
β-actin	Hs03023880_g1	FAM

Annealing temperature: 60°C.

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(ND1), Δ Ct sample indicated the log₂ ratio of mtDNA⁴⁹⁷⁷ vs mtDNA; and Δ Ct reference = Ct Target (modified β -actin)—Ct Endogenous Control (ND1 for β -actin), Δ Ct reference indicated the log₂ ratio of genomic DNA vs mtDNA. Δ Ct sample and Δ Ct reference values were used to evaluate mtDNA⁴⁹⁷⁷ deletion levels.

The common mtDNA deletion of 4,977 bp had breaking points between nucleotides 8461 and 13347, with two direct 13-bp repeats. The fragment was the amplification target between 8461(1st repeat:acctccctcacca) and 13347 bp (2nd repeat:acctaataccca). The grey arrow at the outer circle shows mtDNA deletion of 4,977 bp, and the grey arrow at the inner circle demonstrates the product of the second nested PCR. In the presence of deletion, the amplification product is 358 bp in size, while the wild-type mtDNA yields a product of 5335 bp, which shall not be shown on sequencing. The left corner shows the Sanger sequence that proved the 2nd nested PCR products and confirmed of the 4,977 bp deletions.

Statistical analysis

Pearson’s χ^2 -tests or t-tests were utilized to compare demographic data between the patient and control groups; all significance tests were two-tailed. Association analyses were performed first stratified by sex and then combined. Co-variables included age, sex, hypertension, diabetes mellitus (DM), total cholesterol (TC) level, smoking, and alcohol use. A logistic regression was performed to assess the relationship between mtDNA⁴⁹⁷⁷ and stroke outcome. All data analyses were performed using IBM SPSS Statistics software version 20. The threshold for statistical significance was $p < 0.05$.

Results

Study group characteristics are presented in Table 3. A total of 283 patients and 135 control subjects were included. In the male group, hypertension and smoking were significantly more

Table 3. Demographic data in patients with ischemic stroke and control subjects.

Characteristic	Male group (n = 265)			Female group (n = 153)			p-value for infarct cases vs. control subjects
	Infarct	Control	p-value	Infarct	Control	p-value	
Number	191	74	-	92	61	-	-
Mean age (years)	66.43 ± 12.16	64.95 ± 12.79	0.628	68.40 ± 11.82	66.57 ± 12.56	0.362	0.287
Hypertension (n/%)	131 (68%)	42 (56%)	0.008	70 (76%)	34 (55%)	0.006	< 0.001
Diabetes mellitus (n/%)	63 (32%)	17 (22%)	0.068	44 (47%)	6 (9.8%)	< 0.001	< 0.001
Alcohol use (n/%)	51 (26%)	14 (18%)	0.195	0 (0%)	0 (0%)	-	0.046
Smoking (n/%)	101 (52%)	27 (36%)	0.016	3 (3.2%)	0 (0%)	0.278	< 0.001
Mean BMI (kg/m ²)	25.46 ± 3.55	25.61 ± 3.15	0.779	24.81 ± 3.88	24.63 ± 5.39	0.812	0.745
TC (mg/dl)	178.2 ± 34.40	177.7 ± 34.17	0.873	193.2 ± 42.20	202.4 ± 37.55	0.179	0.149
Initial GCS score ≤ 9	6.1%	-	-	14.7%	-	-	0.375 ^c
30-day MRS score ≥ 3	40%	-	-	50%	-	-	0.042 ^c
4977 deletion (+)	174 (91.1%)	72 (97.3%)	0.079	82 (89.1%)	61 (100%)	0.008	0.002
Δ Ct ^a	2.24 ± 0.86	2.24 ± 0.84	0.998	2.22 ± 0.77	1.94 ± 0.92	0.049	0.155
Δ Ct(4977-ND1) ^b	4.74 ± 2.50	4.92 ± 1.91	0.571	5.07 ± 2.30	5.31 ± 2.04	0.508	0.292

^a Δ Ct = Ct Target (β -actin)—Ct Endogenous Control (ND1 for β -actin)

^b Δ Ct (4977-ND1) = Ct Target (4977 deletion)—Ct Endogenous Control (ND1)

^cp-value for males vs. females in the infarct group

Data are expressed as numbers or mean ± standard error. Comparisons between control subjects and infarction cases were analyzed using χ^2 -tests or t-tests where appropriate. BMI, body-mass index; GCS, Glasgow coma scale; MRS, modified Rankin scale; TC, total cholesterol.

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common in patients with stroke than in control subjects ($p = 0.008$ and $p = 0.016$, respectively). In the female group, hypertension and DM were more common in patients with stroke than in control subjects ($p = 0.006$ and $p < 0.001$, respectively). When the groups were combined, patients with ischemic stroke showed higher prevalence of hypertension ($p < 0.001$), DM ($p < 0.001$), smoking ($p < 0.001$), and alcohol consumption ($p = 0.046$) compared to the control group. There were no significant differences in age, cholesterol level, body mass index, initial Glasgow coma scale (GCS) scores (≤ 9), ΔCt samples, and ΔCt reference values between the sexes. GCS scores were later dichotomized at 8, as this is the clinical cutoff point often used for coma [24]. We thus used an initial GCS score of ≤ 9 as an indicator of initial conscious level and stroke severity. Additionally, higher percentages of mtDNA⁴⁹⁷⁷ were detected in the control group than in the ischemic stroke group (male group: control vs. stroke, 97.3% vs. 91.1%; female group: control vs. stroke, 100% vs. 89.1%; ischemic stroke group vs. control group, $p = 0.002$). The average intra-coefficient of variation (CV) and inter-CV of ΔCt (mtDNA⁴⁹⁷⁷-ND1) is 2.1% and 6.8%, respectively.

Of the study cohort subjects (29 study subjects), 6.94% did not carry mtDNA⁴⁹⁷⁷, including 27 patients with infarction and two healthy control subjects. In these patients, deletion of 4977 was undetectable using TaqMan quantification and confirmed by Sanger sequencing. There was no difference in mean age, stroke risk factors, or ΔCt reference values between participants with and without detectable mtDNA⁴⁹⁷⁷ (Table 4). Participants without detectable mtDNA⁴⁹⁷⁷ had a higher rate of infarction than patients with detectable mtDNA⁴⁹⁷⁷ (male group: 89.5% vs. 70.7%, $p = 0.079$; female group: 100% vs. 57.3%, $p = 0.008$; ischemic stroke group vs. control group, $p = 0.002$) (Table 4).

After adjusting for confounding factors, there was no correlation between mtDNA⁴⁹⁷⁷ amount and infarction risk (odds ratio [OR] = 0.983, 95% confidence interval [CI] = 0.881–1.097, $p = 0.762$), recurrent stroke (OR = 1.106, 95% CI = 0.952–1.285, $p = 0.187$), and stroke severity (OR = 0.953, 95% CI = 0.778–1.168, $p = 0.645$) (Table 5). Consistent with our initial analysis, individuals who did not carry mtDNA⁴⁹⁷⁷ were more likely to have a stroke than individuals with detectable mtDNA⁴⁹⁷⁷ (OR = 0.181, 95% CI = 0.041–0.798, $p = 0.024$).

Table 4. Comparison of patients with and without the 4977 deletion.

Characteristic	Male group (n = 265)			Female group (n = 153)			p-value for subjects with vs. without the 4977 deletion
	4977 deletion	no 4977 deletion	p-value	4977 deletion	no 4977 deletion	p-value	
No. of Infarct/Patient	174/246, 70.7%	7/19, 89.5%	0.079	82/143, 57.3%	10/10, 100%	0.008	0.002
Mean age (years)	66.50 ± 12.30	72.74 ± 0.96	0.166	67.90 ± 12.16	64.40 ± 11.97	0.392	0.135
Hypertension (n/%)	173 (70.3%)	15 (78.9%)	0.425	95 (66.4%)	9 (90%)	0.129	0.123
Diabetes mellitus (n/%)	80 (32.5%)	6 (31.6%)	0.904	47 (32.9%)	3 (30%)	0.840	0.836
Alcohol use (n/%)	65 (26.4%)	8 (42.1%)	0.130	0 (0%)	0 (0%)	-	0.125
Smoking (n/%)	128 (52.0%)	10 (52.6%)	0.914	3 (2.01%)	0 (0%)	0.826	0.847
Mean BMI (kg/m ²)	25.51 ± 3.44	25.18 ± 2.45	0.615	24.84 ± 4.58	22.83 ± 2.91	0.102	0.156
TC (mg/dl)	178.1 ± 34.26	182.7 ± 40.71	0.641	197.7 ± 40.36	184.8 ± 36.63	0.310	0.833
Initial GCS score ≤ 9	6.1%	14%	0.423	14.7%	0	0.351	0.375
30-day MRS score ≥ 3	40%	22%	0.254	50%	100%	0.021	0.462
ΔCt ^a	-2.26 ± 0.86	-1.99 ± 6.80	0.166	-2.12 ± 0.86	-1.93 ± 0.64	0.377	0.098

^a $\Delta Ct = Ct \text{ Target } (\beta\text{-actin}) - Ct \text{ Endogenous Control (ND1 for } \beta\text{-actin)}$

Data are expressed as the number or mean ± standard error. Comparisons between subjects with and without the 4977 deletion were analyzed using χ^2 -tests or t-tests where appropriate. BMI, body-mass index; GCS, Glasgow coma scale; MRS, modified Rankin scale; TC, total cholesterol.

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Table 5. Logistic regression models of mt4977 deletion and stroke prognosis.

Characteristic	Infarction		MRS \geq 3		Recurrent infarcts	
	OR (95% CI)	p-value	OR (95% CI)	p-value	OR (95% CI)	p-value
Δ Ct (4977-ND1) ^a	0.983 (0.881–1.097)	0.762	1.106 (0.952–1.285)	0.187	0.953 (0.778–1.168)	0.645
Presence of 4977 ^b	0.181 (0.041–0.798)	0.024	0.619 (0.195–1.969)	0.417	1.109 (0.279–4.411)	0.883

^aLogistic regression model for Δ Ct (4977-ND1) and stroke prognosis, adjusting for age, sex, TC, diabetes mellitus, hypertension, and smoking.

^bLogistic regression model for presence of the 4977 deletion and stroke prognosis, adjusting for age, sex, TC, diabetes mellitus, hypertension, and smoking. CI, confidence interval; MRS, modified Rankin scale; OR, odds ratio; TC, total cholesterol

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Additionally, the presence or absence of mtDNA⁴⁹⁷⁷ had no effect on stroke prognosis (recurrent stroke: OR = 1.109, 95% CI = 0.279–4.411, p = 0.883; stroke severity: OR = 0.619, 95% CI = 0.195–1.969, p = 0.417).

Discussion

The mtDNA⁴⁹⁷⁷ deletion is particularly relevant to the pathogenesis of atherosclerosis and has previously been reported as a potential predictor of coronary artery disease and stroke; yet, the relationship between mtDNA⁴⁹⁷⁷ and ischemic stroke remains unclear. This study found no correlation between mtDNA⁴⁹⁷⁷ amount and infarction risk, recurrent stroke, or stroke severity in a cohort of patients with acute ischemic stroke. Additionally, the presence or absence of mtDNA⁴⁹⁷⁷ had no effect on stroke prognosis, including recurrent stroke and stroke severity. In our cohort of 283 patients and 135 control subjects, 6.94% of cohort subjects did not carry mtDNA⁴⁹⁷⁷, and there was a significant higher prevalence of stroke in these individuals compared to those with detectable mtDNA⁴⁹⁷⁷. In conclusion, there was no apparent association between mtDNA⁴⁹⁷⁷ deletion and cerebral infarction. Undetectable mtDNA⁴⁹⁷⁷ may be a marker or risk factor for ischemic stroke.

MtDNA⁴⁹⁷⁷ are related to ROS that arise as a by-product of oxidative phosphorylation in mitochondria [25]. MtDNA⁴⁹⁷⁷ occur frequently in tissues of high oxygen demand and low mitotic activity, e.g. neuron cell or myocytes [26]. However, mtDNA⁴⁹⁷⁷ can still be detected in fast replicating cells, such as blood leukocytes, in much lower amounts [25, 26]. Botto et al. reported that mtDNA⁴⁹⁷⁷ can be found in both samples of blood cells and atherosclerotic lesions from patients with CAD [9]. Although mtDNA⁴⁹⁷⁷ in blood could not represent specifically for cerebral infarction, it is still a biomarker for systemic burden of oxidative stress. Because the brain tissue samples from stroke patients are difficult to access, biomarkers in blood is relatively more applicable.

MtDNA is more susceptible to oxidative damage than nuclear DNA [6] because of a limited mitochondrial capacity for DNA repair and the absence of histones for protection [8]. Numerous studies have implicated mtDNA⁴⁹⁷⁷ in various forms of carcinogenesis [27] as well as in aging [28]. mtDNA⁴⁹⁷⁷ is more frequent in the general population than previously thought; it was recently demonstrated that mtDNA⁴⁹⁷⁷ was present in 98.3% of subjects in a cohort of unrelated Chinese participants aged 5 days to 91 years, with an exponential increase in the frequency of mtDNA⁴⁹⁷⁷ with age [18]. This is consistent with the observation of mtDNA⁴⁹⁷⁷ in 93.06% of subjects (389/418) in our study cohort. The discrepancy between earlier and these current findings are likely related to cohorts of a wider age range as well as technological improvements in DNA detection.

MtDNA⁴⁹⁷⁷ in cardiac myocytes and smooth muscle cells in atherosclerotic lesions are important contributors to pathogenesis of coronary artery disease (CAD) [29, 30]. Accumulation of mtDNA⁴⁹⁷⁷ is particularly found in high oxygen demand and low mitotic activity

tissues. Regional accumulation of mtDNA⁴⁹⁷⁷ was higher in the left heart than in the right because the left heart has a higher workload and thus requires higher energy demand [31]. Regarding the hypothetical relationship between mitochondrial damage in atherogenesis and the generation of ROS and ATP synthesis, our data demonstrates that the presence of mtDNA⁴⁹⁷⁷ did not vary in accordance with a history of ischemic stroke. Additionally, mtDNA⁴⁹⁷⁷ levels were not significantly associated with recurrent stroke or stroke prognosis. Our results do not support the notion that mtDNA⁴⁹⁷⁷ plays a major role in cerebral vascular atherosclerosis and cerebral infarction. In one study, mtDNA dysfunction and damage preceded early atherosclerotic changes, while oxidative markers remained normal, suggesting that changes were probably independent of oxidative stress [5]. Moreover, stroke syndromes caused by defects of mtDNA and nuclear DNA may be different, and recent clinical reports suggest that small vessel diseases are associated with diverse nuclear genetic variants [2, 32].

Because accumulation of mtDNA⁴⁹⁷⁷ is found in high oxygen demand and low mitotic activity tissues, such as neuron cells and myocytes, prior CAD models demonstrated increasing mtDNA⁴⁹⁷⁷ and ROS production in coronary artery, aorta, and cardiac myocytes. In contrast, leukocytes are fast replicating with high mitotic activity, which may cause insufficient time for mtDNA⁴⁹⁷⁷ accumulation. Utilizing leukocytes from peripheral blood, our study showed that undetectable mtDNA⁴⁹⁷⁷ in leukocytes may be a biomarker for ischemic stroke. Because the brain tissue samples from stroke patients are difficult to access to confirm the link between undetectable mtDNA⁴⁹⁷⁷ and stroke, further animal model for confirmation is required before utilizing peripheral mtDNA⁴⁹⁷⁷ as a biomarker for ischemic stroke.

A meta-analysis of 38 studies found that proportions of mtDNA⁴⁹⁷⁷ were significantly decreased in cancerous tissue compared to adjacent non-cancerous tissue [33]. Another previous study showed that the frequency of mtDNA⁴⁹⁷⁷ was significantly higher in normal tissue compared to paired cancerous tissues including breast [34, 35], lung [36], esophageal squamous cell carcinoma, gastric [37], and colorectal cancers [23]. The possible mechanism underlying this relationship may be that mtDNA⁴⁹⁷⁷ serves a protective function against the tumor-promoting effects of other somatic mutations [38] or clonal expansion dilution of mtDNA⁴⁹⁷⁷ in tumor tissue during cancer progression. Moreover, mtDNA⁴⁹⁷⁷ might confer a metabolic disadvantage to hyper-proliferating cells and thus eliminate these cells by leading to apoptosis [29]. This may explain our finding that only a low percentage of individuals in our cohort (6.94%) did not carry mtDNA⁴⁹⁷⁷, and the finding that stroke was more prevalent in these individuals.

A limitation of the present study is that patients with cardiovascular problems such as atrial fibrillation and acute coronary syndrome, as well as patients with severe stroke (initial GCS score ≤ 9 ; male: 6.1%, female: 14.7%) were excluded. Second, the small sample size is another limitation of this study. Based on the values of mtDNA⁴⁹⁷⁷ in this study, at the level of 0.05, we will need approximate 1,200 case-control pairs to achieve an adequate power of 80% to detect differences in the level of mtDNA⁴⁹⁷⁷ between the ischemic cases and the control group.

Future work should include a larger and more generalizable cohort to examine correlations between mtDNA⁴⁹⁷⁷ and clinical stroke occurrence or stroke outcome.

Conclusions

MtDNA⁴⁹⁷⁷ does not play a significant role in cerebral infarction. Inability to detect mtDNA⁴⁹⁷⁷ may be a marker or risk factor for ischemic stroke.

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Writing – original draft: Yu-hua Huang.

Writing – review & editing: Yi-Chun Chen.

References

1. Anderson CD, Biffi A, Nalls MA, Devan WJ, Schwab K, Ayres AM, et al. Common variants within oxidative phosphorylation genes influence risk of ischemic stroke and intracerebral hemorrhage. *Stroke; a journal of cerebral circulation*. 2013; 44(3):612–9. Epub 2013/01/31. <https://doi.org/10.1161/strokeaha.112.672089> PMID: [23362085](https://pubmed.ncbi.nlm.nih.gov/23362085/).
2. Borghini A, Cervelli T, Galli A, Andreassi MG. DNA modifications in atherosclerosis: from the past to the future. *Atherosclerosis*. 2013; 230(2):202–9. Epub 2013/10/01. <https://doi.org/10.1016/j.atherosclerosis.2013.07.038> PMID: [24075745](https://pubmed.ncbi.nlm.nih.gov/24075745/).
3. Madamanchi NR, Runge MS. Mitochondrial dysfunction in atherosclerosis. *Circulation research*. 2007; 100(4):460–73. Epub 2007/03/03. <https://doi.org/10.1161/01.RES.0000258450.44413.96> PMID: [17332437](https://pubmed.ncbi.nlm.nih.gov/17332437/).
4. Sobenin IA, Chistiakov DA, Bobryshev YV, Postnov AY, Orekhov AN. Mitochondrial mutations in atherosclerosis: new solutions in research and possible clinical applications. *Current pharmaceutical design*. 2013; 19(33):5942–53. Epub 2013/02/27. PMID: [23438955](https://pubmed.ncbi.nlm.nih.gov/23438955/).
5. Yu E, Calvert PA, Mercer JR, Harrison J, Baker L, Figg NL, et al. Mitochondrial DNA damage can promote atherosclerosis independently of reactive oxygen species through effects on smooth muscle cells and monocytes and correlates with higher-risk plaques in humans. *Circulation*. 2013; 128(7):702–12. Epub 2013/07/12. <https://doi.org/10.1161/CIRCULATIONAHA.113.002271> PMID: [23841983](https://pubmed.ncbi.nlm.nih.gov/23841983/).
6. Yakes FM, Van Houten B. Mitochondrial DNA damage is more extensive and persists longer than nuclear DNA damage in human cells following oxidative stress. *Proceedings of the National Academy of Sciences of the United States of America*. 1997; 94(2):514–9. Epub 1997/01/21. PMID: [9012815](https://pubmed.ncbi.nlm.nih.gov/9012815/).
7. Dimauro S, Davidzon G. Mitochondrial DNA and disease. *Annals of medicine*. 2005; 37(3):222–32. Epub 2005/07/16. <https://doi.org/10.1080/07853890510007368> PMID: [16019721](https://pubmed.ncbi.nlm.nih.gov/16019721/).
8. Agodi A, Barchitta M, Quattrocchi A, Marchese AE, Boffetta P. Folate deficiency is not associated with increased mitochondrial genomic instability: results from dietary intake and lymphocytic mtDNA 4977-bp deletion in healthy young women in Italy. *Mutagenesis*. 2014; 29(2):101–6. Epub 2014/01/15. <https://doi.org/10.1093/mutage/get065> PMID: [24419225](https://pubmed.ncbi.nlm.nih.gov/24419225/).
9. Botto N, Berti S, Manfredi S, Al-Jabri A, Federici C, Clerico A, et al. Detection of mtDNA with 4977 bp deletion in blood cells and atherosclerotic lesions of patients with coronary artery disease. *Mutation research*. 2005; 570(1):81–8. Epub 2005/02/01. <https://doi.org/10.1016/j.mrfmmm.2004.10.003> PMID: [15680405](https://pubmed.ncbi.nlm.nih.gov/15680405/).
10. Fahn HJ, Wang LS, Hsieh RH, Chang SC, Kao SH, Huang MH, et al. Age-related 4,977 bp deletion in human lung mitochondrial DNA. *American journal of respiratory and critical care medicine*. 1996; 154(4 Pt 1):1141–5. Epub 1996/10/01. <https://doi.org/10.1164/ajrccm.154.4.8887618> PMID: [8887618](https://pubmed.ncbi.nlm.nih.gov/8887618/).
11. Lai LP, Tsai CC, Su MJ, Lin JL, Chen YS, Tseng YZ, et al. Atrial fibrillation is associated with accumulation of aging-related common type mitochondrial DNA deletion mutation in human atrial tissue. *Chest*. 2003; 123(2):539–44. Epub 2003/02/11. PMID: [12576378](https://pubmed.ncbi.nlm.nih.gov/12576378/).
12. Hsieh FI, Lien LM, Chen ST, Bai CH, Sun MC, Tseng HP, et al. Get With the Guidelines-Stroke performance indicators: surveillance of stroke care in the Taiwan Stroke Registry: Get With the Guidelines-Stroke in Taiwan. *Circulation*. 2010; 122(11):1116–23. Epub 2010/09/02. <https://doi.org/10.1161/CIRCULATIONAHA.110.936526> PMID: [20805428](https://pubmed.ncbi.nlm.nih.gov/20805428/).
13. Marnane M, Duggan CA, Sheehan OC, Merwick A, Hannon N, Curtin D, et al. Stroke subtype classification to mechanism-specific and undetermined categories by TOAST, A-S-C-O, and causative

- classification system: direct comparison in the North Dublin population stroke study. *Stroke; a journal of cerebral circulation*. 2010; 41(8):1579–86. Epub 2010/07/03. <https://doi.org/10.1161/strokeaha.109.575373> PMID: 20595675.
14. Ren J, Pulakat L, Whaley-Connell A, Sowers JR. Mitochondrial biogenesis in the metabolic syndrome and cardiovascular disease. *Journal of molecular medicine (Berlin, Germany)*. 2010; 88(10):993–1001. Epub 2010/08/21. <https://doi.org/10.1007/s00109-010-0663-9> PMID: 20725711
 15. Peinado JR, Diaz-Ruiz A, Fruhbeck G, Malagon MM. Mitochondria in metabolic disease: getting clues from proteomic studies. *Proteomics*. 2014; 14(4–5):452–66. Epub 2013/12/18. <https://doi.org/10.1002/pmic.201300376> PMID: 24339000.
 16. Knight-Lozano CA, Young CG, Burow DL, Hu ZY, Uyeminami D, Pinkerton KE, et al. Cigarette smoke exposure and hypercholesterolemia increase mitochondrial damage in cardiovascular tissues. *Circulation*. 2002; 105(7):849–54. Epub 2002/02/21. PMID: 11854126.
 17. Meissner C, Bruse P, Mohamed SA, Schulz A, Warnk H, Storm T, et al. The 4977 bp deletion of mitochondrial DNA in human skeletal muscle, heart and different areas of the brain: a useful biomarker or more? *Experimental gerontology*. 2008; 43(7):645–52. Epub 2008/04/29. <https://doi.org/10.1016/j.exger.2008.03.004> PMID: 18439778.
 18. Zheng Y, Luo X, Zhu J, Zhang X, Zhu Y, Cheng H, et al. Mitochondrial DNA 4977 bp deletion is a common phenomenon in hair and increases with age. *Bosnian journal of basic medical sciences*. 2012; 12(3):187–92. Epub 2012/09/04. <https://doi.org/10.17305/bjbm.2012.2480> PMID: 22938547.
 19. DiMauro S, Schon EA. Mitochondrial disorders in the nervous system. *Annual review of neuroscience*. 2008; 31:91–123. Epub 2008/03/13. <https://doi.org/10.1146/annurev.neuro.30.051606.094302> PMID: 18333761.
 20. Deschauer M, Tennant S, Rokicka A, He L, Kraya T, Turnbull DM, et al. MELAS associated with mutations in the POLG1 gene. *Neurology*. 2007; 68(20):1741–2. Epub 2007/05/16. <https://doi.org/10.1212/01.wnl.0000261929.92478.3e> PMID: 17502560.
 21. Andreatza AC, Shao L, Wang JF, Young LT. Mitochondrial complex I activity and oxidative damage to mitochondrial proteins in the prefrontal cortex of patients with bipolar disorder. *Archives of general psychiatry*. 2010; 67(4):360–8. Epub 2010/04/07. <https://doi.org/10.1001/archgenpsychiatry.2010.22> PMID: 20368511.
 22. Adams HP Jr., Bendixen BH, Kappelle LJ, Biller J, Love BB, Gordon DL, et al. Classification of subtype of acute ischemic stroke. Definitions for use in a multicenter clinical trial. TOAST. Trial of Org 10172 in Acute Stroke Treatment. *Stroke; a journal of cerebral circulation*. 1993; 24(1):35–41. Epub 1993/01/01. PMID: 7678184.
 23. Chen T, He J, Shen L, Fang H, Nie H, Jin T, et al. The mitochondrial DNA 4,977-bp deletion and its implication in copy number alteration in colorectal cancer. *BMC medical genetics*. 2011; 12:8. Epub 2011/01/15. <https://doi.org/10.1186/1471-2350-12-8> PMID: 21232124.
 24. Tsao JW, Hemphill JC 3rd, Johnston SC, Smith WS, Bonovich DC. Initial Glasgow Coma Scale score predicts outcome following thrombolysis for posterior circulation stroke. *Arch Neurol*. 2005; 62(7):1126–9. Epub 2005/07/13. <https://doi.org/10.1001/archneur.62.7.1126> PMID: 16009770.
 25. Yan MH, Wang X, Zhu X. Mitochondrial defects and oxidative stress in Alzheimer disease and Parkinson disease. *Free radical biology & medicine*. 2013; 62:90–101. Epub 2012/12/04. <https://doi.org/10.1016/j.freeradbiomed.2012.11.014> PMID: 23200807.
 26. von Wurmb-Schwark N, Schwark T, Caliebe A, Drenske C, Nikolaus S, Schreiber S, et al. Low level of the mtDNA(4977) deletion in blood of exceptionally old individuals. *Mechanisms of ageing and development*. 2010; 131(3):179–84. Epub 2010/02/02. <https://doi.org/10.1016/j.mad.2010.01.005> PMID: 20117127.
 27. Brandon M, Baldi P, Wallace DC. Mitochondrial mutations in cancer. *Oncogene*. 2006; 25(34):4647–62. Epub 2006/08/08. <https://doi.org/10.1038/sj.onc.1209607> PMID: 16892079.
 28. Pavicic WH, Richard SM. Correlation analysis between mtDNA 4977-bp deletion and ageing. *Mutation research*. 2009; 670(1–2):99–102. Epub 2009/08/04. <https://doi.org/10.1016/j.mrfmmm.2009.07.009> PMID: 19646455.
 29. Bogliolo M, Izzotti A, De Flora S, Carli C, Abbondandolo A, Degan P. Detection of the '4977 bp' mitochondrial DNA deletion in human atherosclerotic lesions. *Mutagenesis*. 1999; 14(1):77–82. Epub 1999/09/04. PMID: 10474826.
 30. Sabatino L, Botto N, Borghini A, Turchi S, Andreassi MG. Development of a new multiplex quantitative real-time PCR assay for the detection of the mtDNA(4977) deletion in coronary artery disease patients: a link with telomere shortening. *Environmental and molecular mutagenesis*. 2013; 54(5):299–307. Epub 2013/05/25. <https://doi.org/10.1002/em.21783> PMID: 23703697.

31. Corral-Debrinski M, Shoffner JM, Lott MT, Wallace DC. Association of mitochondrial DNA damage with aging and coronary atherosclerotic heart disease. *Mutation research*. 1992; 275(3–6):169–80. Epub 1992/09/01. PMID: [1383759](#).
32. Anderson CD, Biffi A, Rahman R, Ross OA, Jagiella JM, Kissela B, et al. Common mitochondrial sequence variants in ischemic stroke. *Annals of neurology*. 2011; 69(3):471–80. Epub 2010/09/15. <https://doi.org/10.1002/ana.22108> PMID: [20839239](#).
33. Nie H, Shu H, Vartak R, Milstein AC, Mo Y, Hu X, et al. Mitochondrial common deletion, a potential biomarker for cancer occurrence, is selected against in cancer background: a meta-analysis of 38 studies. *PloS one*. 2013; 8(7):e67953. Epub 2013/07/19. <https://doi.org/10.1371/journal.pone.0067953> PMID: [23861839](#).
34. Ye C, Shu XO, Wen W, Pierce L, Courtney R, Gao YT, et al. Quantitative analysis of mitochondrial DNA 4977-bp deletion in sporadic breast cancer and benign breast diseases. *Breast cancer research and treatment*. 2008; 108(3):427–34. Epub 2007/06/02. <https://doi.org/10.1007/s10549-007-9613-9> PMID: [17541740](#).
35. Dimberg J, Hong TT, Nguyen LTT, Skarstedt M, Lofgren S, Matussek A. Common 4977 bp deletion and novel alterations in mitochondrial DNA in Vietnamese patients with breast cancer. *SpringerPlus*. 2015; 4:58. Epub 2015/02/13. <https://doi.org/10.1186/s40064-015-0843-8> PMID: [25674508](#).
36. Dai JG, Xiao YB, Min JX, Zhang GQ, Yao K, Zhou RJ. Mitochondrial DNA 4977 BP deletion mutations in lung carcinoma. *Indian journal of cancer*. 2006; 43(1):20–5. Epub 2006/06/10. PMID: [16763358](#).
37. Wu CW, Yin PH, Hung WY, Li AF, Li SH, Chi CW, et al. Mitochondrial DNA mutations and mitochondrial DNA depletion in gastric cancer. *Genes, chromosomes & cancer*. 2005; 44(1):19–28. Epub 2005/05/14. <https://doi.org/10.1002/gcc.20213> PMID: [15892105](#).
38. Dani SU, Dani MA, Simpson AJ. The common mitochondrial DNA deletion Δ mtDNA(4977): shedding new light to the concept of a tumor suppressor mutation. *Medical hypotheses*. 2003; 61(1):60–3. Epub 2003/06/05. PMID: [12781642](#).