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Presence of mutation m.14484T>C in a Chinese family with maternally inherited essential hypertension but no expression of LHON

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ABSTRACT

Essential hypertension (EH, MIM 145500) is the most common cardiovascular disease and affects one-quarter of the world's adult population. Families with EH in a mode of maternal transmission have been occasionally observed in clinical settings and suggested an involvement of mitochondrial DNA (mtDNA) mutation. We aimed to characterize the role of mtDNA mutation in EH. We reported a large Han Chinese family with a maternally inherited EH and an extraordinarily high percentage of sudden death mainly in affected females. Analysis of the entire mtDNA genome of the proband identified a homoplasmic primary mutation m.14484T>C for Leber's hereditary optic neuropathy (LHON), along with several variants indicating haplogroup F1 status. Intriguingly, no maternal member in this family had LHON though they all harbored m.14484T>C. The arterial stiffness of the members carrying mutation m.14484T>C was significantly increased than that of non-maternal members without this mutation. No environmental factor (including age, sex, smoking, diabetes, hyperlipidemia) was correlated with the decreased aortic elastic properties observed in affected members. Mitochondrial respiration rate and membrane potential ($\Delta\Psi_m$) were significantly reduced in lymphoblastoid cell lines established from affected members carrying m.14484T>C when compared to control cell lines ($P<0.05$). There was an elevation of reactive oxygen species and a compensatory increase of mitochondrial mass in mutant cell lines. Our results suggest that m.14484T>C causes EH under certain circumstance. This study provides a paradigm for diverse phenotypes of the primary LHON mutation and suggests for the necessity of routine cardiac evaluation in patients with the primary LHON mutation.

1. Introduction

Essential hypertension (EH, MIM 145500) is one of the most common cardiovascular diseases and acts as a significant risk factor for heart attack, stroke and end-stage renal disease [1,2]. It causes a heavy global burden and around 7.6 million people die of this disease each year [3]. Though the exact molecular pathogenesis and mode of inheritance of EH have not been fully elucidated, genetic factor was demonstrated to be actively involved in the onset of EH according to family and epidemiologic studies [4,5]. Previous studies showed that about 35–55% of EH cases were associated with mitochondrial dysfunction [6,7]. Clinical observation of many EH cases from families with a maternal transmission of the disease suggested that mitochondrial DNA (mtDNA) mutations might account for the onset of EH [6–8]. There are several reports for identifying pathogenic mtDNA mutations in patients with hypertension [9–13], but the exact mechanism has not been sufficiently elucidated [14].

One clinical feature of mitochondrial disease is the complex symptom caused by the same mtDNA pathogenic mutation. For instance, mutation m.3243A>G could lead to a variety of human disorders such as mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS) and diabetes [15,16]. Many factors including mtDNA mutation/background, nuclear genes and environmental factors were actively involved in the development of maternally inherited diseases [17].

In this study, we characterized a large Han Chinese family with maternally inherited EH and mutation m.14484T>C. We found that the maternal members in this family presented no acute or subacute central visual loss but only hypertension. We provided further experimental evidence to show that the onset of hypertension in this family was caused by the LHON mutation m.14484T>C.

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2. Materials and methods

A five-generation Han Chinese family (Fig. 1) with maternally inherited EH (family EH10) was diagnosed at the Department of Cardiology in the Calmette Hospital, Kunming Medical University. Among the 36 subjects in this family, 10 individuals died (5 hypertensives and 5 normotensives); living individuals included 10 maternal members (4 hypertensives and 6 normotensives) and 16 non-maternal members (16 normotensives). We divided all the living individuals into case group (maternally-related members) and control group (non-maternal members) for comparison. Physical measurements for each member were conducted following the WHO MONICA Project standards. Individuals with EH were diagnosed according to the Joint National Committee VI criteria [18]. We sequenced the entire mtDNA genome of proband (IV:1) using the method described in our previous study [19] and defined the (potentially) pathogenic mutation in the matriline by a phylogenetic approach [20]. We further quantified the heteroplasmic or homoplasmic status of the identified pathogenic mutation in hair root, saliva and whole blood from the proband, as well as in all maternally-related relatives of the proband and all non-maternal individuals in this pedigree.

We established lymphoblastoid cell lines for three maternally related (affected members III:5 and IV:1 and asymptomatic member V:1) and three unrelated healthy individuals (C1, C2, and C3). We quantified oxygen consumption rates (OCR), intracellular reactive oxygen species (ROS), mitochondrial membrane potential ($\Delta\Psi m$) and mitochondrial mass in each cell line.

Continuous variables were expressed as mean ± SD, and discrete variables in groups were expressed as frequency. Data were tested for normality using the one-sample Kolmogorov–Smirnov test (2-tailed). Continuous variables between the two groups (maternally-related members and non-maternal members) were analyzed by both non-parametric (Mann–Whitney *U* test and two-sample Kolmogorov–Smirnov test) and parametric (independent samples *t*-test) methods. Because of the limited sample size for each group (10 versus 16), non-parametric tests were preferred in this study. The parametric tests were also performed as they have more statistical power for the same number of observations under the assumption of normality or the assumption of homogeneity of variance. The Levene's test was applied to evaluate the equality of variances. Fisher's exact test (two-tailed) was used to compare the rates of the patient's gender, smoking and retinal microvascular structural changes (RMSC). Analysis of covariance (age, sex and BMI acting as simultaneous covariates) was used to compare biochemical test values, current visual acuity, ambulatory blood pressure monitoring (ABPM) parameters and echocardiographic parameters of the maternally-related members and non-maternal members. All statistical analyses were performed using SPSS 13.0 (SPSS Inc., Chicago, IL, USA), and $P<0.05$ was considered as statistically significant.

A detailed Methods section is available in the Online Data Supplement. Written informed consents were obtained from all participants prior to the study. This study was approved by the institutional review boards of the Calmette Hospital and the Kunming Institute of Zoology.

3. Results

3.1. Clinical features of the family with EH

The proband (IV:1) was a 52-year-old woman and suffered from hypertension at age 47. Her highest blood pressure [systolic blood pressure (SBP)/diastolic blood pressure (DBP)] was 170/110 mm Hg in the past 5 years. The proband visited the Calmette Hospital for further clinical evaluation in 2009 because of intermittent dizziness and headache. After being treated by calcium channel blocker (CCB) and angiotensin-receptor blocker, her blood pressure reduced to a normal range (110–130/70–80 mm Hg). All clinical indexes were normal except for ambulatory arterial stiffness index (AASI, 0.6), symmetric ambulatory arterial stiffness index (S-AASI, 0.4) and pulse wave velocity (PWV, 12.9 m/s), which showed abnormal values and suggested for declined aorta's compliance (Fig. 2). The echocardiogram showed E/A ratio abnormality, which was one of the most common cardiac abnormalities in hypertension.

The proband had slight myopia at both eyes, and visual acuity was 0.6 in the right eye and 0.9 in the left eye. Ophthalmoscopic examination showed an abnormal pattern of chronic hypertensive retinopathy: the arteriole-to-venule ratio of retinal vessels was reduced to 1:3 and arteriovenous nicking was seen at the upper temporal quadrant, despite that both of her optic disks were normal. Examinations for intraocular pressure, visual field and color vision showed no sign of abnormality.

Comprehensively medical examination for all members in this family showed no signs of a hearing problem, muscular diseases and neurological disorders. Examination of electrocardiogram (ECG) and cranial magnetic resonance imaging (MRI) revealed no abnormality. All family members (except for proband IV:1) did not receive any antihypertensive or other cardiovascular drugs before and during their participation in this study. Measurement of aortic PWV and PP (pulse pressure) showed an increased arterial stiffness in the maternally-related offspring (Fig. 2 and Table S1). Nine of 17 maternal members in the pedigree expressed hypertension and the affected male to female ratio was 1:3.5 (Fig. 1 and Table 1). Among them, five died of sudden death (stroke and acute myocardial infarction) and the percentage of sudden death was 100%. The mean age at onset in this family was 45.5 years (range

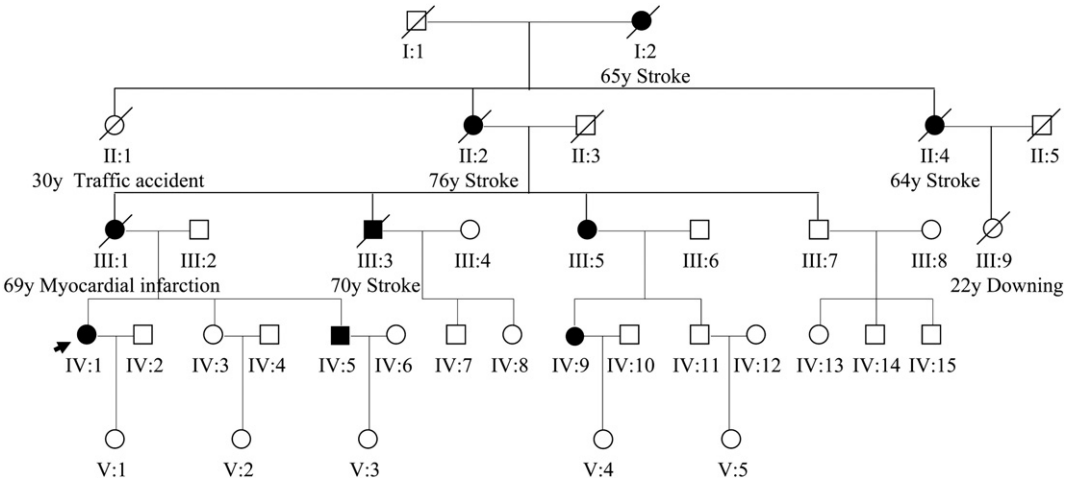


Fig. 1. Pedigree information for a Chinese family with maternally inherited EH and m.14484T>C. Affected individuals were marked by filled symbols. The proband that was analyzed for the complete mtDNA sequence was marked by an arrow. The age at death of each deceased member was listed below the sample name.

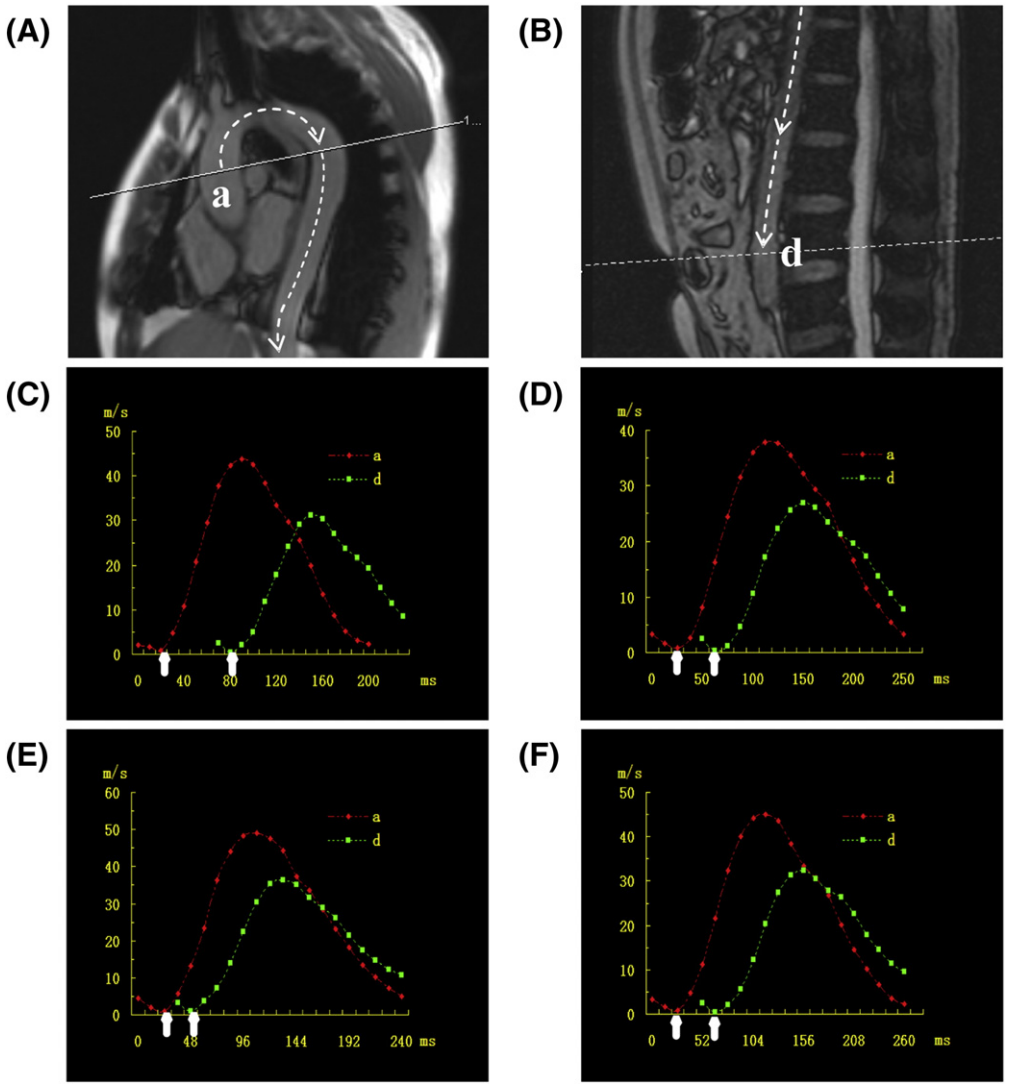


Fig. 2. Imaging of MR cine phase-contrast and the velocity–time curves of ascending aorta and the abdominal aorta. The position imaging of ascending aorta (A) and abdominal aorta (B) were measured by MR cine phase-contrast. Non-maternal member IV:8 (48 years old, male) of this family had good compliance and slow PWV (7.2 m/s) (C), whereas the proband (IV:1) had bad compliance and fast PWV (12.9 m/s) (D). Maternal members III:5 and IV:3 of the proband all had bad compliance and fast PWV (E: III:5, 14.5 m/s; F: IV:3, 11.0 m/s). "a"—Level of ascending aorta, which is 1.5 cm above the aortic valve; "d"—level of abdominal aorta, which is 1.5 cm above the common iliac bifurcation; "ad" is the distance between the ascending aorta and the abdominal aorta. The beginning points of the time–speed curve of ascending aorta and abdominal aorta are marked by arrows.

Table 1
Clinical information of maternally-related members in family EH10.

Subject	Gender	Age at test (years)	Age of onset (years)	Age of death (years)	BPT (SBP/DBP) (mm Hg)	HCBPMR (SBP/DBP) (mm Hg)	Cause of death
I:2	F	–	Unknown	65	–	Unknown	Stroke
II:1	F	–	–	30	–	Unknown	Accident
II:2	F	–	50	76	–	180/90	Stroke
II:4	F	–	48	64	–	180/90	Stroke
III:1	F	–	40	69	–	180/110	AMI
III:3	M	–	40	70	–	180/100	Stroke
III:5	F	73	45	–	160/90	170/90	–
III:7	M	69	–	–	105/64	120/70	–
III:9	F	–	–	22	–	Unknown	Drowning
IV:1	F	52	47	–	120/80	170/110	–
IV:3	F	50	–	–	96/60	125/70	–
IV:5	M	47	48	–	145/90	160/100	–
IV:9	F	44	46	–	135/85	156/100	–
IV:11	M	42	–	–	106/64	120/68	–
V:1	F	27	–	–	114/72	120/75	–
V:2	F	19	–	–	100/60	110/62	–
V:4	F	24	–	–	100/70	115/75	–

F: female; M: male; SBP: systolic blood pressure; DBP: diastolic blood pressure; AMI: acute myocardial infarction; HCBPMR: the highest casual blood pressure of medical record; BPT: blood pressure of test.

40 to 50). Probands III:1 (an obstetrician–gynecologist) and III:3 (a surgeon) had a relatively early onset of EH (both at age 40), possibly burdened by work load and stress. The mean onset age of affected members in generation III (41.7 years) was smaller than that of generation II (49.0 years) or IV (47.0 years); note that this estimation was based on a very limited number of individuals (Tables 1 and S1). The penetrance of EH in the maternally-related members was 52.9% (9/17), and it increased to 64.3% (9/14) after we excluded younger members in the fifth generation (mean age 23.3 ± 4.0 years).

We compared the ambulatory blood pressure monitoring (ABPM) parameters, PWV and biochemical test values between the maternal and non-maternal members by using both non-parametric and parametric tests (with adjustment for age, sex and body mass index [BMI]). As shown in Table 2 and Table S2, the maternally-related members had a significantly higher value of 24 h pulse pressure (24hPP) ($P<0.05$), day pulse pressure (dPP) ($P<0.02$), night pulse pressure (nPP) ($P<0.01$), S-AASI ($P<0.02$), AASI ($P<0.02$) and PWV ($P<0.02$) than the non-maternal members. There was statistical difference for SBP of daytime, nighttime and 24-hour ($P<0.05$) between the two groups when the effects of age, sex, and BMI were taken into consideration. Other factors including age, gender, smoking, BMI, waist hip ratio (WHR), fasting blood sugar, blood lipids, uric acid, and renal function were similar between the two groups ($P>0.05$). Although the differences of eye vision and rate of retinal microvascular structural changes (RMSC)

Table 2
Clinical features of all 26 members in family EH10.

Parameter	Maternal members ($n=10$)	Non-maternal members ($n=16$)	*P*-value[a]	*P*-value[b]	*P*-value[c]
Demographic information					
Age, years	44.70 ± 17.91	49.81 ± 16.94	0.471	0.580	0.992
Female (%)	7 (70)	8 (50)	0.428	0.428	0.428
Smoking (%)	4 (40)	7 (44)	1.000	1.000	1.000
BMI, kg/m^2	21.59 ± 2.54	22.45 ± 2.13	0.362	0.329	0.637
WHR	0.79 ± 0.09	0.82 ± 0.07	0.340	0.178	0.314
Biochemical test values					
FBS, mmol/L	4.49 ± 0.38	4.70 ± 0.28	0.119	0.193	0.438
TC, mmol/L	4.54 ± 0.32	4.68 ± 0.71	0.501	0.579	0.394
TG, mmol/L	1.18 ± 0.23	1.51 ± 0.72	0.100	0.126	0.216
HDL, mmol/L	1.43 ± 0.23	1.32 ± 0.31	0.326	0.343	0.637
LDL, mmol/L	2.53 ± 0.47	2.90 ± 0.79	0.140	0.235	0.534
UA, μmol/L	255.50 ± 71.76	265.44 ± 103.99	0.794	0.854	0.944
BUN, mmol/L	5.40 ± 0.94	5.79 ± 0.66	0.221	0.303	0.314
Cr, μmol/L	101.20 ± 16.88	97.88 ± 15.60	0.613	0.895	0.992
Optical examinations					
Current visual acuity (OD)	0.72 ± 0.40	0.67 ± 0.40	0.754	0.749	0.992
Current visual acuity (OS)	0.78 ± 0.33	0.69 ± 0.35	0.511	0.557	0.966
Prevalence of RMSC, n (%)	7 (70)	5 (31)	0.105	0.105	0.105
ABPM parameters					
24hSBP, mm Hg	110.30 ± 12.07	102.75 ± 7.26	0.097	0.096	0.485
24hDBP, mm Hg	64.60 ± 4.17	68.69 ± 6.31	0.083	0.090	0.144
24hPP, mm Hg	45.70 ± 10.12	34.06 ± 3.86	0.005*	0.001*	0.034*
dSBP, mm Hg	114.70 ± 12.24	107.88 ± 7.17	0.134	0.162	0.352
dDBP, mm Hg	67.50 ± 4.45	71.44 ± 6.29	0.098	0.064	0.314
dPP, mm Hg	47.20 ± 9.19	36.44 ± 3.95	0.005*	0.001*	0.013*
nSBP, mm Hg	102.70 ± 11.00	96.13 ± 6.65	0.111	0.107	0.352
nDBP, mm Hg	60.20 ± 3.97	64.44 ± 6.85	0.089	0.125	0.189
nPP, mm Hg	42.50 ± 9.07	31.69 ± 4.63	0.004*	0.001*	0.007*
S-AASI	0.32 ± 0.13	0.20 ± 0.06	0.014*	0.007*	0.013*
AASI	0.46 ± 0.14	0.30 ± 0.07	0.008*	0.005*	0.013*
24hHR, bpm	76.20 ± 7.61	74.88 ± 10.00	0.723	0.833	0.992
Aorta's compliance					
PWV, m/sec	10.76 ± 1.97	7.89 ± 1.10	0.001*	0.001*	0.016*
Echocardiographic parameters					
LV_d, mm	42.30 ± 4.40	39.88 ± 2.03	0.129	0.137	0.165
LA, mm	32.00 ± 6.45	28.99 ± 2.06	0.183	0.039*	0.013*
IVS, mm	9.59 ± 1.68	8.45 ± 0.48	0.063	0.090	0.005*
LVPW, mm	9.14 ± 1.49	8.06 ± 0.66	0.053	0.101	0.024*
LVMI, g/m^2	95.61 ± 34.66	65.94 ± 10.81	0.025*	0.061	0.005*
EF, %	64.04 ± 2.78	63.66 ± 2.81	0.741	0.654	0.999
E, m/s	0.90 ± 0.09	0.93 ± 0.13	0.467	0.561	0.836
A, m/s	0.81 ± 0.14	0.73 ± 0.09	0.139	0.154	0.485
E/A	1.14 ± 0.20	1.28 ± 0.14	0.069	0.178	0.278

BMI: body mass index; WHR: waist hip ratio; FBS: fasting blood sugar; TC: total cholesterol; TG: triglyceride; HDL: high-density lipoprotein; LDL: low-density lipoprotein; UA: urea acid; BUN: blood urea nitrogen; Cr: creatine; OD: right eye; OS: left eye; RMSC: retinal microvascular structural changes; ABPM: ambulatory blood pressure monitoring; 24hSBP: 24 h systolic blood pressure; 24hDBP: 24 h diastolic blood pressure; 24hPP: 24 h pulse pressure; dSBP: day systolic blood pressure; dDBP: day diastolic blood pressure; dPP: day pulse pressure; nSBP: night systolic blood pressure; nDBP: night diastolic blood pressure; nPP: night pulse pressure; S-AASI: symmetric ambulatory arterial stiffness index; AASI: ambulatory arterial stiffness index; 24hHR: 24 h heart rate; PWV: pulse wave velocity; LV_d: left ventricular diastolic diameter; LA: left atrium diameter; IVS: interventricular septum thickness; LVPW: left ventricular posterior wall thickness; LVMI: left ventricular mass index; EF: ejection fraction; E: early mitral inflow velocity; A: late mitral inflow velocity.

* A *P* value<0.05 was marked by a star.

[a] Independent samples *t*-test.

[b] Mann–Whitney *U* test and.

[c] Two-sample Kolmogorov–Smirnov test was used for continuous variables. For discrete variables, Fisher's exact test (two-tailed) was used.

between the two groups were not statistically different ($P>0.05$), maternally-related subjects had a higher prevalence of retinal microvascular signs (Table 2). All these observations suggested that arterial structural abnormality of mother–offspring units was more severe than that of non-mother–offspring units.

Echocardiographic parameters of all members were shown in Table S3. The maternally-related members displayed a significantly higher level of left ventricular mass index (LVMI) (two-sample Kolmogorov–Smirnov test, $P=0.005$; independent samples t-test, $P=0.025$; adjusted $P<0.001$) (Table 2 and Table S2) compared to non-maternal members. Significant differences for left ventricular diastolic diameter (LV_d), left atrium diameter (LA), interventricular septum thickness (IVS), left ventricular posterior wall thickness (LVPW), and E/A were observed between the two groups when effects of age, sex, and BMI were considered ($P<0.05$) (Table S2), suggesting hypertension and arterial stiffness-associated cardiac remodeling.

3.2. Presence of m.14484T>C in the matriline

Analysis of the complete mtDNA sequence of the proband identified a total of 30 homoplasmic variants relative to the revised Cambridge reference sequence (rCRS) [21], in which 23 were haplogroup-characteristic variants and suggested it belongs to haplogroup F1b'd (Fig. 3). Among 7 private variants in this matriline, four (m.309insCC, m.523–524del, m.16183A>C and m.16519T>C) were located in the control region, three were located in the coding region (m.2389C>T in the *MT-RNR2* gene, m.3398T>C in the *MT-ND1* gene, and m.14484T>C in the *MT-ND6* gene). All these private variants were previously reported and had no potential pathogenicity except for m.14484T>C, which was one of the well-known primary LHON mutations. Mutation m.14484T>C was homoplasmic in hair root, saliva and whole blood from the proband (Fig. S1). This mutation was presented in all maternally-related relatives of the proband but was absent in all non-maternal individuals (Figs. S1 and S2). We presented a sequence variation of the proband, together with some related mtDNAs which belonged to the same haplogroup in a tree. Apparently, there was no clear evidence for a synergistic effect between m.14484T>C and other private variants in this matriline, as this mtDNA did not harbor any private variants on its terminal branch in the tree (Fig. 3). The complete mtDNA sequences of the proband and a healthy control (C3) belonging to haplogroup F1a1c were deposited in GenBank under accession nos. JN133516–JN133517.

3.3. Alteration of mitochondrial function in mutant cells

To evaluate mitochondrial function of lymphoblastoid cell lines established from three maternally-related members with m.14484T>C and three unrelated healthy controls, we used a Clark electrode to measure oxygen consumption rate (OCR) in live cells. The mutant cell lines exhibited significantly lower OCR than control cell lines (Fig. 4 and Table S7). We added different respiratory inhibitors to determine which respiratory complex was the pronounced one being affected. We employed ATP-coupled respiration, basal mitochondrial respiration and maximal respiratory capacity to represent the activities of complex V, complex I and the overall mitochondrial oxygen consumption capacity dependent on the mitochondrial membrane potential, respectively. The OCR value of each respective state was normalized to the initial value before adding the inhibitor, which was arbitrarily set to 1. Surprisingly, all three mitochondrial respiration parameters decreased significantly in mutant cell lines ($P<0.05$) (Fig. 4B and Table S7). As maximal respiratory capacity reflected the permeability of a mitochondrial outer membrane ($\Delta\Psi_m$), we measured membrane potential in cell lines with and without m.14484T>C. Concordant with the OCR result, the mean value of $\Delta\Psi_m$ in mutant cells decreased remarkably (up to 87.9%) compared to that of control cells ($P<0.05$, Fig. 5A–B and Table S7).

We further tested the alteration of reactive oxygen species (ROS) and mitochondrial mass in cell lines with and without m.14484T>C,

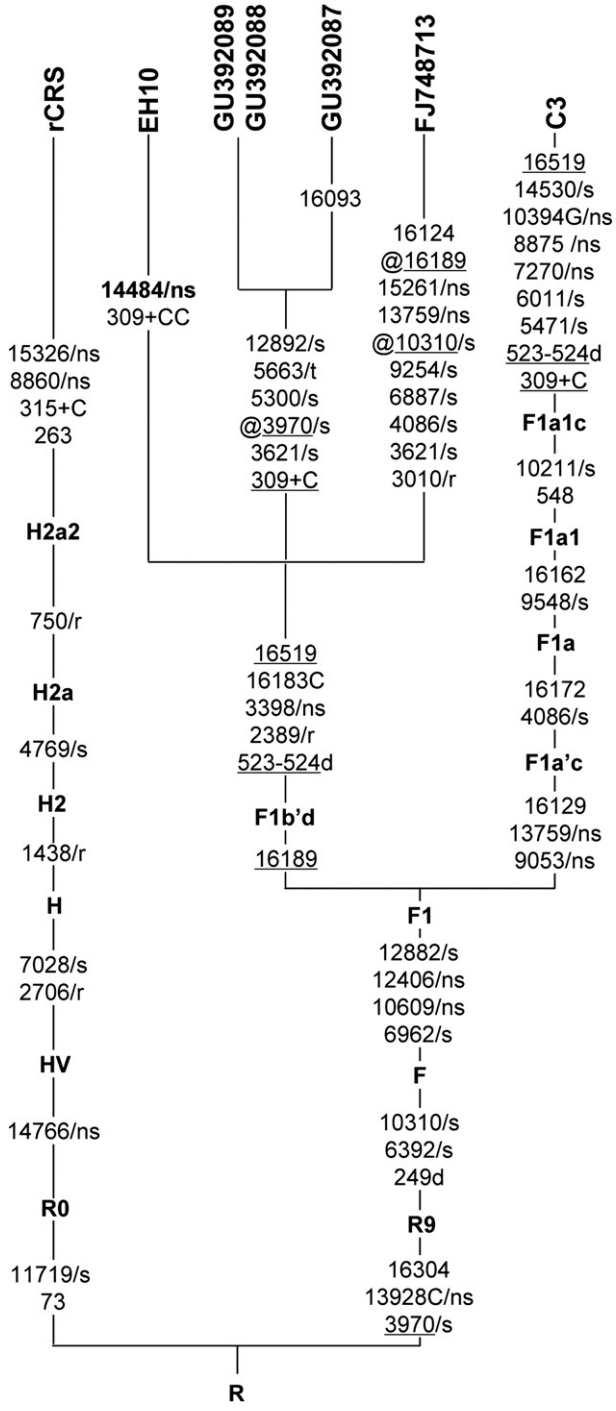


Fig. 3. Classification tree of the proband mtDNA, four near-matched sequences from published sources and one healthy donor. Sequences GU392087, GU392088, GU392089 and FJ748713 were from GenBank. Healthy donor C3 (28 years old, male) was newly determined in this study. All sequence variations were scored relative to the revised Cambridge reference sequence (rCRS) [21]. The order of mutations on each uninterrupted branch section is arbitrary. Recurrent variations are underlined; back mutations are underlined and marked by "@". Suffix "G" refers to transversion and "+C" indicates an insertion of cytosine. Previously confirmed pathogenic mutations are in bold. The synonymous and non-synonymous coding-region variants in each mtDNA are further denoted by "/s" and "/ns", respectively. Variations in the ribosomal RNA genes are denoted by "/r".

which are sensitive to mitochondrial dysfunction. The average ROS level of mutant cells was higher than that of control cells (Fig. 5C–D and Table S7), with an increase of up to 140.6%. Intriguingly, we also observed an elevation of mean value of mitochondrial mass (up to 37.4%, albeit the difference was not statistically significant) in all mutant cell

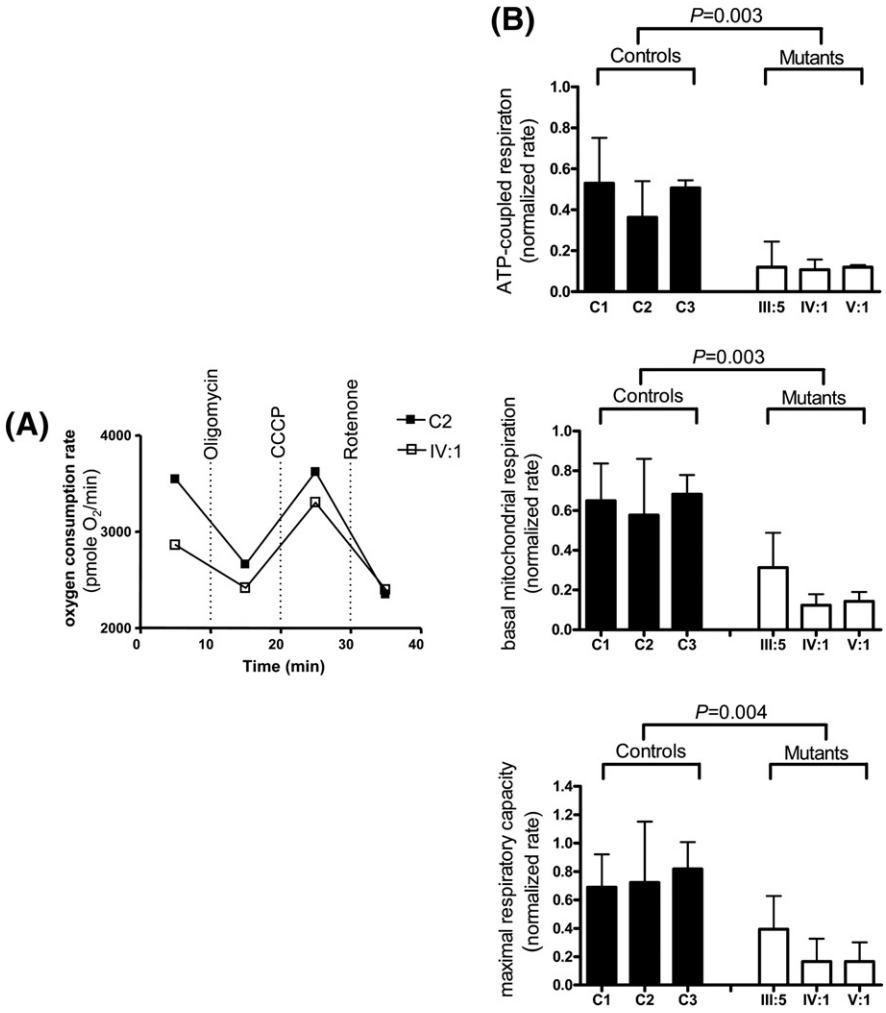


Fig. 4. Mutation m.14484T>C affects mitochondrial respiratory functions. (A) Representative diagram of absolute oxygen consumption rates (OCR, pmol per minute per 3×10^6 cells) in cell lines of a healthy control (C2) and a patient with m.14484T>C (IV:1). Vertical dashed lines indicate the time of adding the indicated drugs. (B) Different respiratory parameters in the control cell lines (C1, C2 and C3) and cell lines with m.14484T>C (III:5, IV:1 and V:1). Rates were normalized to the oxygen consumption rate prior to the treatment of drugs, which was arbitrarily set to 1. The bar represents mean ± SD of the rates collected from three independent measurements.

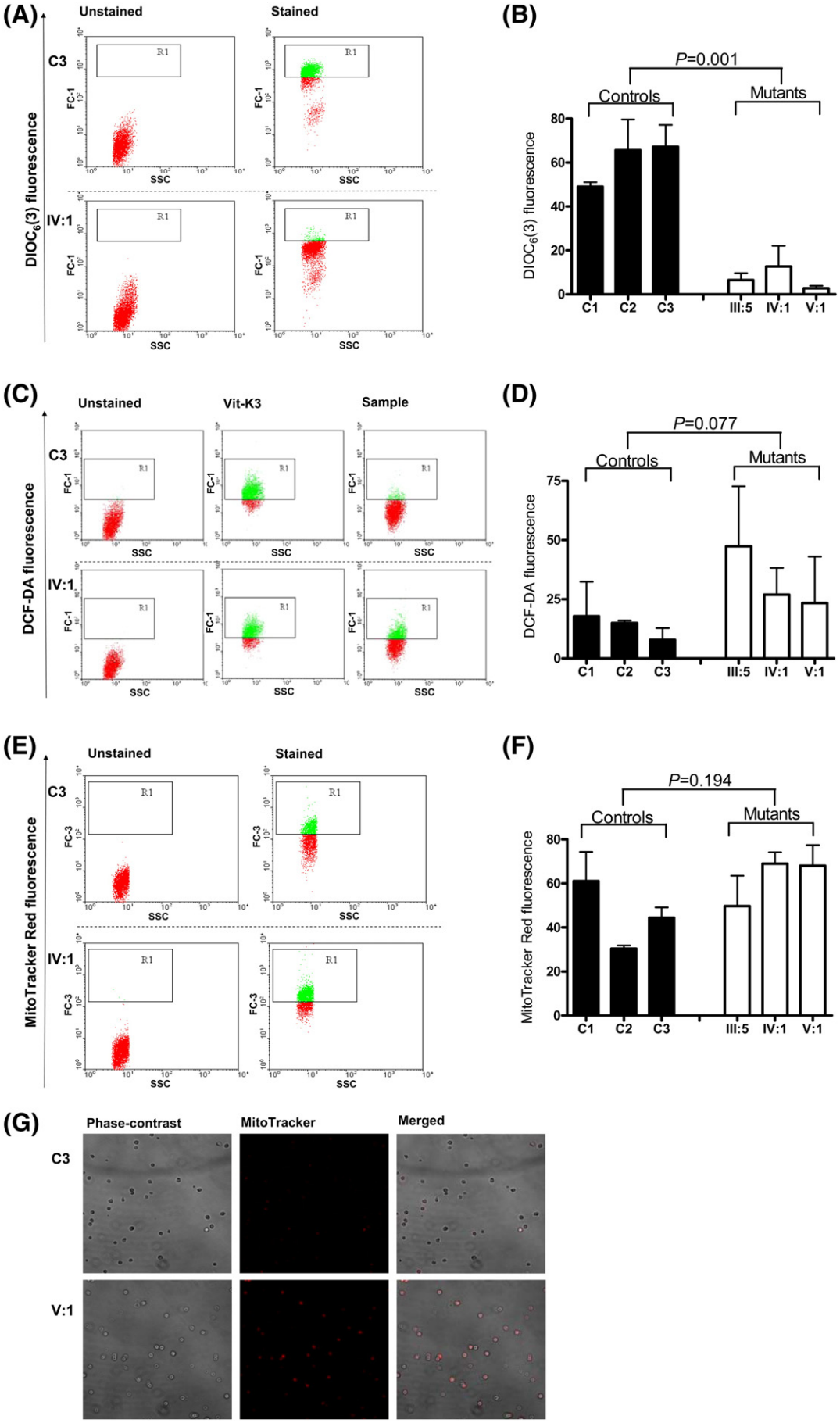
lines (Fig. 5E–G and Table S7), suggesting for a potential compensation for mitochondrial dysfunction in the presence of m.14484T>C. Accordingly, we observed no essential difference for an mtDNA copy number (Fig. S3) and the overall cellular ATP levels (Fig. S4) between mutant and control cell lines.

4. Discussion

Accumulating evidence suggested that mitochondrial damage and dysfunction were actively involved in cardiovascular disease [22,23]. In this study, we performed the clinical, genetic, and molecular characterization of a large family with maternal hereditary hypertension and LHON mutation m.14484T>C. Clinical examination and evaluation showed that the parameters related to vascular aging (including PP, S-AASI, AASI and PWV) of the maternally-related members presented significantly higher values than those of non-maternal members. In particular, four maternally-related members died of hemorrhagic stroke and one died of acute myocardial infarction, indicating problems with vascular remodeling in this family. The aortic stiffness, as one of the main causes of increased blood pressure [24], was evident in affected members (Fig. 3; Tables 2 and S1). Our observation indicated that the primary increase in large artery stiffness caused by mutation m.14484T>C preceded the development of hypertension [25]. However, affected members of this family were not isolated systolic hypertension, indicating that structural abnormality of small arteries may also play a role in EH. More families are required to solidify our speculation.

The striking clinical feature of this family is that all maternally-related members had no apparent sign for LHON but EH, despite that they had

Fig. 5. Alterations in ΔΨm, intracellular ROS and mitochondrial mass. (A) Representative result of flow cytofluorometric analysis for ΔΨm quantification. Cell lines established from IV:1 and healthy control (C3) was analyzed by DiOC6(3) staining. R1 gates the polarized population. (B) The relative ΔΨm values of cell lines from three normal individuals (C1, C2 and C3) and three affected members with m.14484T>C (III:5, IV:1 and V:1). Each bar represents mean ± SD of three independent measurements. The mean value of ΔΨm in mutant cells was decreased by 87.9% compared to that of the control cell lines. (C) Representative staining of intracellular ROS using DCF-DA in cell lines derived from normal individual C3 and patient IV:1 R1 gates the ROS positive population. Vitamin K3 (Vit-K3), which induced intracellular ROS increase was used as a positive control. Cells were incubated with 160 μM Vit-K3 at 37 °C for 3 h before the final staining. (D) Quantification of relative ROS level in cell lines of three normal individuals and three members of the family with m.14484T>C mutation. Bars represent mean ± SD of five independent experiments. In all experiments, each cell line was cultured and analyzed in duplicated wells. (E) Representative diagram for quantification of mitochondrial mass. Cell lines from IV:1 and from control (C3) were stained by MitoTracker Red FM and were analyzed by flow cytofluorometric analysis. R1 gates the positive red fluorescence population. (F) Mutant cells lines had a compensatory increased mitochondrial mass compared to that of control cell lines. Results are mean ± SD of two independent experiments with triplicate samples. (G) Mitochondrial mass of cell lines from V:1 and from control (C3) were stained by MitoTracker Red FM and were analyzed by confocal microscopy; depicted in this figure is the original magnification × 100.


(A)
Unstained
Stained
C3
IV:1
R1
FC-1
SSC
DIOC6(3) fluorescence
(B)
P=0.001
Controls
Mutants
DIOC6(3) fluorescence
C1
C2
C3
III:5
IV:1
V:1
(C)
Unstained
Vit-K3
Sample
DCF-DA fluorescence
(D)
P=0.077
(E)
MitoTracker Red fluorescence
FC-3
(F)
P=0.194
(G)
Phase-contrast
MitoTracker
Merged
V:1

m.14484T>C (Fig. S1). Although there are studies reporting that LHON patients expressed cardiac or vascular abnormalities, such as pre-excitation syndrome [26,27], myocardial thickening, left ventricular noncompaction (LVNC), dilated heart [28,29], mild degenerative cardiomyopathy [30], high aortic stiffness indexes [31] and intracranial arteriovenous malformation [32], there is no report for the presence of primary LHON mutation m.14484T>C in a family with EH but without clinical expression of LHON to date. Analyses of demographic data and biochemical test values of all available members in this EH family suggested that no environmental factor, but hereditary factor, accounted for the maternal transmission of the disease. Determination of the entire mtDNA genome showed that no other pathogenic mutation, except for m.14484T>C, would account for hypertension (Fig. 3). Note that this family had a non-synonymous variant m.3398T>C, which was said to be pathogenic in previous studies [33,34]. This variant existed in different populations worldwide and was one of characteristic motifs for haplogroups I2a1 and M65b [35]. By comparing to four near-matched sequences from GenBank and generated in this study, we found that m.3398T>C, together with m.2389 C>T, define a novel subhaplogroup of F1b'd in Chinese. Therefore, variant m.3398T>C should be best categorized as a polymorphism. Why the primary LHON mutation m.14484T>C did not cause vision loss but hypertension in this family is a riddle. Nikoskelainen et al. [36] found that only a few LHON mutation carriers with microangiopathy finally developed optic neuropathy, this incomplete penetrance could partially explain why no LHON was observed in this family. It should be mentioned that the matriline of this family belonged to haplogroup F, which was said to be a protective factor for LHON carriers with m.14484T>C [37] and m.11778 G>A [38], and this matrilineal background may relieve the deleterious effect of pathogenic mtDNA mutation on the eye.

Many mtDNA mutations have been reported to be associated with hypertension or cardiovascular diseases [9–12]. One plausible explanation for the pathogenicity of deleterious mtDNA mutation in hypertension is that it causes a decrease of energy production, overproduction of ROS, metabolic imbalance, and disturbed signal transduction, which finally initiated hypertension, atherosclerosis, apoptosis, and necrosis [39]. Dysfunction of mitochondrial respiratory chain decreased electrochemical potential gradient and impaired ATP synthesis, which further destroyed systolic/diastolic functions in vascular smooth muscle cell (VSMC) [40,41]. Elevated ROS induced vascular endothelial cell (VEC) senescence [42,43] and was involved in the development of hypertension and atherosclerosis [22,44]. We were unable to collect VSMC and VEC from the living affected members in this family. However, by using lymphoblastoid cell lines derived from family members and healthy controls, we observed a reduction of respiration rate (including ATP-coupled respiration, maximal respiratory capacity and basal mitochondrial respiration) and $\Delta\Psi_m$, and an increase of ROS level in mutant cell lines. Along with these alterations, there was a substantial increase of mitochondrial mass in mutant cells, which compensated cellular ATP production and mtDNA content in the presence of m.14484T>C. All these lines of evidence supported for mitochondrial defects owing to m.14484T>C. The ROS levels in mutant cells from symptomatic patients (III:5, 47.44% and IV:1, 26.95%) and the asymptomatic patient (V:1, 23.40%) were substantially increased compared to those of control cells (C1, 17.82%; C2, 14.99%; C3, 7.83%). We speculated that an increase of ROS generation and a loss of mitochondrial membrane potential in the presence of m.14484T>C would contribute to endothelial dysfunction, which finally caused impaired elasticity and contractility of the aorta [41,45].

One intriguing observation is that half of the maternally-related members with m.14484T>C presented EH at the time of this study. The incomplete penetrance of EH suggested a potential influence of the nuclear genetic background. Indeed, cells from the asymptomatic member V:1 had increased ROS and decreased mitochondrial membrane potential (Fig. 5 and Table S7), despite the absence of EH and young age (28 years old). Further study, with a proper balance of the different nuclear genetic backgrounds of cases and controls, should be carried out to define the effect of the nuclear background on clinical expression of m.14484T>C. In addition, epigenetic modifications (e.g. gene methylation, acetylation) to genes involved with adverse vessel remodeling, membrane lipid homeostasis and myocardial conduction properties [46,47] should be closely evaluated in future studies, to identify the nuclear genes and other molecular modifications that are actively involved in this process.

In summary, the current report for the presence of m.14484T>C in a Chinese family with maternally inherited EH but without LHON provides a paradigm for diverse phenotypes of the primary LHON mutation. We show several lines of evidence that m.14484T>C caused mitochondrial dysfunction in cells from affected members and this may be the underlying mechanism for hypertension in this family. Given the absence of an overt phenotype in some carriers there is a potential complexity that may also involve mitochondrial genes and other molecular modifications. Patients with the primary LHON mutation should routinely undergo cardiac evaluation of aortic compliance and blood pressure. Our study also provides a new clue for a matrilineal genetic factor of arterial stiffening and vascular aging. Lifestyle intervention or pharmacotherapy that retards and/or reverses age-related vessel remodeling and stiffness may represent a potential target for treatment and prevention of maternally inherited EH.

Conflict of interest

None declared.

Acknowledgements

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Appendix A. Supplementary data

Supplementary material is available at Journal online. Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2012.06.010.

References

[1] J. He, P.K. Whelton, Epidemiology and prevention of hypertension, Med. Clin. North Am. 81 (1997) 1077–1097.
[2] I. Hajjar, J.M. Kotchen, T.A. Kotchen, Hypertension: trends in prevalence, incidence, and control, Annu. Rev. Public Health 27 (2006) 465–490.
[3] C.M.M. Lawes, S. Vander Hoorn, A. Rodgers, International Society of Hypertension, Global burden of blood-pressure-related disease, 2001, Lancet 371 (2008) 1513–1518.
[4] H. Snieder, G.A. Harshfield, F.A. Treiber, Heritability of blood pressure and hemodynamics in African- and European-American youth, Hypertension 41 (2003) 1196–1201.
[5] R.R. Williams, S.C. Hunt, S.J. Hasstedt, P.N. Hopkins, L.L. Wu, T.D. Berry, B.M. Stults, G.K. Barlow, M.C. Schumacher, R.P. Lifton, J.M. Lalouel, Are there interactions and relations between genetic and environmental-factors predisposing to high blood-pressure, Hypertension 18 (1991) 29–37.
[6] Q. Yang, S.K. Kim, F.Z. Sun, J. Cui, M.G. Larson, R.S. Vasan, D. Levy, F. Schwartz, Maternal influence on blood pressure suggests involvement of mitochondrial DNA in the pathogenesis of hypertension: the Framingham Heart Study, J. Hypertens. 25 (2007) 2067–2073.
[7] F.Z. Sun, J. Cui, H. Gavras, F. Schwartz, A novel class of tests for the detection of mitochondrial DNA-mutation involvement in diseases, Am. J. Hum. Genet. 72 (2003) 1515–1526.
[8] A.L. DeStefano, H. Gavras, N. Heard-Costa, M. Bursztyn, A. Manolis, L.A. Farrer, C.T. Baldwin, I. Gavras, F. Schwartz, Maternal component in the familial aggregation of hypertension, Clin. Genet. 60 (2001) 13–21.
[9] B. Watson, M.A. Khan, R.A. Desmond, S. Bergman, Mitochondrial DNA mutations in black Americans with hypertension-associated end-stage renal disease, Am. J. Kidney Dis. 38 (2001) 529–536.
[10] F. Schwartz, A. Duka, F.Z. Sun, J. Cui, A. Manolis, H. Gavras, Mitochondrial genome mutations in hypertensive individuals, Am. J. Hypertens. 17 (2004) 629–635.
[11] F.H. Wilson, A. Hariri, A. Farhi, H.Y. Zhao, K.F. Petersen, H.R. Toka, C. Nelson-Williams, K.M. Raja, C.M. Kashgarian, G.I. Shulman, S.J. Scheinman, R.P.

Lifton, A cluster of metabolic defects caused by mutation in a mitochondrial tRNA, Science 306 (2004) 1190–1194.
[12] S. Wang, R. Li, A. Fettermann, Z. Li, Y. Qian, Y. Liu, X. Wang, A. Zhou, J.Q. Mo, L. Yang, P. Jiang, A. Taschner, W. Rossmanith, M.X. Guan, Maternally inherited essential hypertension is associated with the novel 4263A>G mutation in the mitochondrial tRNAIle gene in a large Han Chinese family, Circ. Res. 108 (2011) 862–870.
[13] H.Y. Zhu, S.W. Wang, L.J. Martin, L. Liu, Y.H. Li, R. Chen, L. Wang, M.L. Zhang, D.W. Benson, The role of mitochondrial genome in essential hypertension in a Chinese Han population, Eur. J. Hum. Genet. 17 (2009) 1501–1506.
[14] A.J. Marian, Mitochondrial genetics and human systemic hypertension, Circ. Res. 108 (2011) 784–786.
[15] Y. Goto, I. Nonaka, S. Horai, A mutation in the tRNA(Leu)(UUR) gene associated with the MELAS subgroup of mitochondrial encephalomyopathies, Nature 348 (1990) 651–653.
[16] J.A. Maassen, G.M. Janssen, L.M. 't Hart, Molecular mechanisms of mitochondrial diabetes (MIDD), Ann. Med. 37 (2005) 213–221.
[17] H.A. Tuppen, E.L. Blakely, D.M. Turnbull, R.W. Taylor, Mitochondrial DNA mutations and human disease, Biochim. Biophys. Acta 1797 (2010) 113–128.
[18] The sixth report of the Joint National Committee on prevention, detection, evaluation, and treatment of high blood pressure, Arch. Intern. Med. 157 (1997) 2413–2446.
[19] H.W. Wang, X.Y. Jia, Y.L. Ji, Q.P. Kong, Q.J. Zhang, Y.G. Yao, Y.P. Zhang, Strikingly different penetrance of LHON in two Chinese families with primary mutation G11778A is independent of mtDNA haplogroup background and secondary mutation G13708A, Mutat. Res. 643 (2008) 48–53.
[20] Y. Ji, A.M. Zhang, X. Jia, Y.P. Zhang, X. Xiao, S. Li, X. Guo, H.J. Bandelt, Q. Zhang, Y.G. Yao, Mitochondrial DNA haplogroups M7b1'2 and M8a affect clinical expression of Leber hereditary optic neuropathy in Chinese families with the m.11778G>A mutation, Am. J. Hum. Genet. 83 (2008) 760–768.
[21] R.M. Andrews, I. Kubacka, P.F. Chinnery, R.N. Lightowlers, D.M. Turnbull, N. Howell, Reanalysis and revision of the Cambridge reference sequence for human mitochondrial DNA, Nat. Genet. 23 (1999) 147.
[22] J. Gutierrez, S.W. Ballinger, V.M. Darley-Usmar, A. Landar, Free radicals, mitochondria, and oxidized lipids: the emerging role in signal transduction in vascular cells, Circ. Res. 99 (2006) 924–932.
[23] S.W. Ballinger, Mitochondrial dysfunction in cardiovascular disease, Free Radic. Biol. Med. 38 (2005) 1278–1295.
[24] M.F. O'Rourke, J.A. Staessen, C. Vlachopoulos, D. Duprez, G.E. Plante, Clinical applications of arterial stiffness; definitions and reference values, Am. J. Hypertens. 15 (2002) 426–444.
[25] E.G. Lakatta, D. Levy, Arterial and cardiac aging: major shareholders in cardiovascular disease enterprises: Part I: Aging arteries: a “set up” for vascular disease, Circulation 107 (2003) 139–146.
[26] E.K. Nikoskelainen, M.L. Savontaus, K. Huoponen, K. Antila, J. Hartiala, Pre-excitation syndrome in Leber's hereditary optic neuropathy, Lancet 344 (1994) 857–858.
[27] Y. Mashima, K. Kigasawa, H. Hasegawa, M. Tani, Y. Oguchi, High incidence of pre-excitation syndrome in Japanese families with Leber's hereditary optic neuropathy, Clin. Genet. 50 (1996) 535–537.
[28] P. Sorajja, M.G. Sweeney, R. Chalmers, B. Sachdev, P. Syrris, M. Hanna, N.D. Wood, W.J. McKenna, P.M. Elliott, Cardiac abnormalities in patients with Leber's hereditary optic neuropathy, Heart 89 (2003) 791–792.
[29] J. Finsterer, C. Stollberger, C. Prainer, A. Hochwarter, Lone noncompaction in Leber's hereditary optic neuropathy, Acta Cardiol. 59 (2004) 187–190.
[30] F.C. Rose, A.N. Bowden, P.M. Bowden, The heart in Leber's optic atrophy, Br. J. Ophthalmol. 54 (1970) 388–393.
[31] A. Nemes, I.F. De Coo, L. Spruijt, H.J. Smeets, P.F. Chinnery, O.I. Soliman, M.L. Geleijnse, F.J. Ten Cate, Is there alteration in aortic stiffness in Leber hereditary optic neuropathy? Eur. J. Ophthalmol. 18 (2008) 309–312.
[32] J. Fujitake, H. Mizuta, H. Fujii, Y. Ishikawa, K. Sasamoto, Y. Goto, I. Nonaka, Y. Tatsuoka, Leber's hereditary optic neuropathy with intracranial arteriovenous malformation: a case report, Acta Neurol. Belg. 102 (2002) 82–86.
[33] S. Tang, A. Batra, Y. Zhang, E.S. Ebenroth, T. Huang, Left ventricular noncompaction is associated with mutations in the mitochondrial genome, Mitochondrion 10 (2010) 350–357.
[34] M. Jaksch, S. Hofmann, P. Kaufhold, B. Obermaier-Kusser, S. Zierz, K.D. Gerbitz, A novel combination of mitochondrial tRNA and ND1 gene mutations in a syndrome with MELAS, cardiomyopathy, and diabetes mellitus, Hum. Mutat. 7 (1996) 358–360.
[35] M. van Oven, M. Kayser, Updated comprehensive phylogenetic tree of global human mitochondrial DNA variation, Hum. Mutat. 30 (2009) E386–E394.
[36] E.K. Nikoskelainen, K. Huoponen, V. Juvonen, T. Lamminen, K. Nummelin, M.L. Savontaus, Ophthalmologic findings in Leber hereditary optic neuropathy, with special reference to mtDNA mutations, Ophthalmology 103 (1996) 504–514.
[37] D. Yu, X. Jia, A.M. Zhang, S. Li, Y. Zou, Q. Zhang, Y.G. Yao, Mitochondrial DNA sequence variation and haplogroup distribution in Chinese patients with LHON and m.14484T>C, PLoS One 5 (2010) e13426.
[38] A.M. Zhang, X. Jia, R. Bi, A. Salas, S. Li, X. Xiao, P. Wang, X. Guo, Q.P. Kong, Q. Zhang, Y.G. Yao, Mitochondrial DNA haplogroup background affects LHON, but not suspected LHON, in Chinese patients, PLoS One 6 (2011) e27750.
[39] L.I. Rachek, V.I. Grishko, S.P. Ledoux, G.L. Wilson, Role of nitric oxide-induced mtDNA damage in mitochondrial dysfunction and apoptosis, Free Radic. Biol. Med. 40 (2006) 754–762.
[40] Y.V. Postnov, S.N. Orlov, Y.Y. Budnikov, A.D. Doroschuk, A.Y. Postnov, Mitochondrial energy conversion disturbance with decrease in ATP production as a source of systemic arterial hypertension, Pathophysiology 14 (2007) 195–204.
[41] Y. Taniyama, K.K. Griendling, Reactive oxygen species in the vasculature: molecular and cellular mechanisms, Hypertension 42 (2003) 1075–1081.
[42] S.W. Ballinger, C. Patterson, C.A. Knight-Lozano, D.L. Burow, C.A. Conklin, Z. Hu, J. Reuf, C. Horaist, R. Lebovitz, G.C. Hunter, K. McIntyre, M.S. Runge, Mitochondrial integrity and function in atherogenesis, Circulation 106 (2002) 544–549.
[43] H. Unterluggauer, B. Hampel, W. Zwerschke, P. Jansen-Durr, Senescence-associated cell death of human endothelial cells: the role of oxidative stress, Exp. Gerontol. 38 (2003) 1149–1160.
[44] G.M. Puddu, E. Cravero, G. Arnone, A. Muscari, P. Puddu, Molecular aspects of atherogenesis: new insights and unsolved questions, J. Biomed. Sci. 12 (2005) 839–853.
[45] P. Puddu, G.M. Puddu, E. Cravero, S. De Pascalis, A. Muscari, The putative role of mitochondrial dysfunction in hypertension, Clin. Exp. Hypertens. 29 (2007) 427–434.
[46] R.M. Millis, Epigenetics and hypertension, Curr. Hypertens. Rep. 13 (2011) 21–28.
[47] M.P. Turunen, E. Aavik, S. Yla-Herttuala, Epigenetics and atherosclerosis, Biochim. Biophys. Acta 1790 (2009) 886–891.

ONLINE SUPPLEMENT

PRESENCE OF MUTATION m.14484T>C IN A CHINESE FAMILY WITH MATERNALLY INHERITED ESSENTIAL HYPERTENSION BUT NO EXPRESSION OF LHON

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Materials and Methods

Subjects

A five-generation Han Chinese family (Fig. 1) with maternally inherited EH (family EH10) was diagnosed at the Department of Cardiology in the Calmette Hospital, Kunming Medical College on November 2009. There are a total of 36 individuals in this family. We performed the latest follow-up physical examination for this family on November 2011. Among them, 10 individuals died (5 hypertensives and 5 normotensives); living individuals included 10 maternal members (4 hypertensives and 6 normotensives) and 16 non-maternal members (16 normotensives). Because all patients in this family are maternally-related members, we divided all living individuals into case group (maternally-related members) and control group (non-maternal members) for comparison.

Written informed consents were obtained from all participants prior to the study. This study was approved by the institutional review boards of the Calmette Hospital and the Kunming Institute of Zoology.

Clinical examination

Members of this family were interviewed, and comprehensive health and life questionnaires were performed for each member. Physical measurements for each member were conducted following the WHO MONICA Project standards. Blood pressure of each participant was measured by using a mercury sphygmomanometer with a standardized fashion. Individuals with EH were diagnosed according to the Joint National Committee VI criteria [1]. Twenty-four hours ambulatory blood pressure monitoring (ABPM) was measured non-invasively by using the Spacelab 90207 or 90217 devices (Spacelab, CA, USA). The ABPM data was recorded every 15 min during the daytime (6:00–23:00) and every 30 min during the nighttime (23:00–6:00). We estimated symmetric ambulatory arterial stiffness index (S-AASI) and ambulatory arterial stiffness index (AASI) using the approach by Gavish *et al* [2]. The transthoracic echocardiographic examinations for all subjects were made during rest in left lateral decubitus position, using HP SONOS 5500 (HP, CA, USA) and standard echocardiographic evaluation techniques. Left atrium (LA) size was measured at the end of ventricular systole; left ventricular (LV) internal dimension and LV posterior wall (LVPW) and interventricular septal (IVS) thicknesses were measured at the end of ventricular diastole according to methods established by the American Society of Echocardiography [3]. LV mass (LVM) was estimated by using the anatomically validated equation reported by Devereux *et al* [4]. The LV mass index (LVMI) was calculated by dividing LVM by body surface area. Systolic function including the left ventricular ejection fraction (EF) and diastolic function including ratio (E/A ratio) of the early peak (E) to late peak (A) diastolic mitral inflow velocity were obtained. Aortic pulse wave velocity (PWV) was measured and evaluated by 1.5-T magnetic resonance imaging (MRI) with MR cine phase-contrast (Siemens, Munich, Germany). PWV was calculated as the ratio of the distance between the ascending aorta and the abdominal aorta (m) over the time (s) required for the pulse wave to travel from the ascending aorta to the abdominal aorta. Aortic length used for calculating PWV included the distances from 1.5 cm above the aortic valve to the top of the aortic arch and from the top of the aortic arch to 1.5 cm above the bifurcation of the common iliac. Blood chemistry tests for fasting blood sugar, total cholesterol, triglycerides, high density lipoprotein, low density lipoprotein, blood urea nitrogen, creatinine and urea acid were done by automatic biochemistry analyzer (OLYMPUS AU-5400, Tokyo, Japan). Other special examinations included cranial MRI and electrocardiogram (ECG) (GE Marquette MAC 5500, CT, USA).

Ophthalmoscopic examination was carried out and analyzed blindly by two ophthalmologists. All subjects underwent an evaluation of their ocular history, and had a complete ophthalmic examination, including visual acuities, intraocular pressure, visual field, color vision, slit-lamp biomicroscopy with fundus examination and direct ophthalmoscopy. Retinal arteriolosclerotic

signs were assessed following the Scheie classification [5] and prevalence of retinal microvascular structural changes (RMSC) were calculated by the following formula: subjects with the light reflex changes of retinal arteriolosclerosis (from grade 1 to grade 4 of Scheie classification) / all subjects (from grade 0 to grade 4 of Scheie classification) × 100 (%).

At the beginning of collection of clinical data for this family, we only performed physical examination for the proband and her maternally-related relatives, which include a measurement of blood pressure, blood biochemical test, electrocardiogram (ECG), 24-hours ABPM, and transthoracic echocardiography. Optical examinations, cranial MRI and PWV were measured and evaluated when we found that the maternally-related family members carried mutation m.14484T>C. The purpose of these examinations was to confirm whether there is a coincidence of LHON phenotype and intracranial arteriovenous malformation (IAM), and we used the golden standard of arterial stiffness – PWV to verify the outcome of two new arterial stiffness indexes AASI and S-AASI.

mtDNA genome analysis

The genomic DNA was extracted from whole blood, saliva and hair root by using the commercial DNA Isolation Kit (Tiangen Biotech Co., Beijing, China) or the lysis method for extracting genomic DNA from single cells [6], respectively. The entire mtDNA genome of proband (IV: 1) was amplified and sequenced as described in our previous study [7]. The mtDNA pathogenic mutation m.14484T>C was further detected in the above three tissues/specimens from proband and her maternal relatives. For non-maternal relatives of the proband and three healthy controls that were used for establishing lymphoblastoid cell lines, genomic DNA extracted from blood was used for screening m.14484T>C by sequencing.

Sequences were analyzed by using the DNASTAR software package (DNASTAR Inc., WI, USA). Sequence variants were scored relative to the revised Cambridge Reference Sequence (rCRS) [8]. Haplogroup classification was performed according to the updated world mtDNA tree [9] and was verified by using MitoTool (http://www.mitotool.org) [10]. We defined the potentially "novel" status of certain mtDNA variant(s) by an exhausted database search following the guidelines described in our recent study [11]. The phylogenetic approach was used to define the (potentially) pathogenic mutation in the matriline.[12] mtDNA sequence variation of the proband and a healthy control sample (C3), together with the rCRS [8] and four mtDNA sequences from GenBank (Accession numbers GU392087, GU392088, GU392089 and FJ748713), were presented in a tree profile following the same approach in our previous study [12].

Cell line establishment

Blood samples from three maternally-related (affected members III:5 and IV:1 and asymptomatic member V:1) and three unrelated healthy individuals (C1, C2, and C3) were collected for establishing lymphoblastoid cell lines. Three maternally-related members were chosen to represent members from different generations in family EH10, which shared same mtDNA haplogroup F1. The control samples were age-matched with the patients and belonged to different mtDNA haplogroups (C1 contains 16140-16182C-16183C-16189-16217-16242-16274-16335-16519-73-146-150-263-315+C-(523-524)delAC-709-750 relative to the rCRS [8] and belongs to haplogroup B4c1b2; C2 contains 16223-16260-16290-16319-16355-64-73-146-195-235-263-309+C-315+C-(523-524)delAC-663-750 and belongs to haplogroup A; C3 was sequenced for the entire mtDNA genome [c.f. Figure 3 in the main text] and belongs to haplogroup F1a1c). All these three healthy donors were confirmed to be absent of m.14484T>C. In particular, subject C3 shared same mtDNA haplogroup F1 with the affected members from this EH family, which is better to demonstrate potential effect of m.14484T>C, instead of haplogroup effect, in the functional characterization assays.

Lymphoblastoid cell lines were immortalized using the Epstein-Barr virus. Cells were

maintained in RPMI 1640 supplemented with 10% fetal bovine serum (Gibco, USA).

Detection of cellular reactive oxygen species (ROS) and mitochondrial membrane potential ($\Delta\Psi_m$)

We used 0.1 μM DCF-DA (Sigma-Aldrich) for measuring the cellular ROS and 20 nM $DiOC_6(3)$ (Sigma-Aldrich) for $\Delta\Psi_m$ quantification. In brief, cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum for 24 h. About $1x10^6$ cells were incubated with DCF-DA or $DiOC_6(3)$ in a total volume of 100 μL culture medium at 37°C for 30 min. After the stained cells were pelleted and washed with 500 μL PBS, the fluorescence of the resuspended cells (in 100 μL PBS) was analyzed by using flow cytometry (BD, Vantage SE, USA). The fluorescence intensities of DCF-DA and $DiOC_6(3)$ were monitored at 535 nm and 501 nm, respectively.

Determination of mitochondrial mass and mtDNA copy number

Change in mitochondrial mass was assessed by using MitoTracker Red FM (Molecular Probe, USA). Cells were pelleted and resuspended in pre-warm medium containing 100nM MitoTracker Red FM at 37°C for 30 min, then were washed with pre-warm medium and PBS. The stained cells were examined by using flow cytometry at 644 nm. Cells were further imaged under a laser scanning confocal microscopy (LSCM) (ZEISS, LSM 510 META, Germany).

Mitochondrial DNA copy number in the six lymphoblastoid cell lines was measured by using real-time quantitative PCR according to our recent study [13]. Briefly, mtDNA copy number was normalized to a single copy nuclear β-globin gene. The following primer pairs were used for real-time PCR analysis: for β-globin gene, HBB502f (5'-CTATGGGACGCTTGATGT-3') / HBB614r (5'-GCAATCATTCGTCTGTTT-3'); for mtDNA, L394 (5'-CACCAGCCTAACCAGATTTC-3')/H475 (5'-GGGTTGTATTGATGAGATTAGT-3'). The ratio of mtDNA to nuclear DNA reflects the relative mtDNA content within cells. The real-time PCR amplification was performed using SYBR® Premix Ex TaqTM II kit (TaKaRa Biotechnology Co. Ltd., Japan) according the manufacturer's manual on MyiQ2 (BioRad Laboratories, Hercules, CA, USA).

Determination of cellular ATP level

Cellular ATP measurements were performed by using the ATP Determination Kit (Invitrogen, USA) according to the manufacture's manual. Cells were seeded in 6-well plates and grown for 24 h. Approximate $1x10^7$ cells were lysed in 100 μL lysis buffer with protease inhibitor. After incubating at 4 °C for 10 min, cell lysate were centrifuged at 12000 g for 10 min and supernatant was collected. An aliquot of 10 μL was added to 90 μL assay solution and the ATP content was measured on GloMax 96 Luminometer (Promega).

Oxygen consumption

Cellular oxygen consumption was measured using intact cells and a Clark-type oxygen sensor (Hansatech instruments, England) at 25°C. The respiratory parameters were calculated as described before [14]. Briefly, 3×10^6 cells were analyzed in growth culture medium. After having recorded the respiration of cells in culture medium for 10 min, ATP synthase inhibitor oligomycin (1 μM) was added into the chamber to collect the ATP-coupled respiration for 10 min. Then, mitochondrial uncoupler CCCP (10 μM) was added and the maximal respiratory capacity of mitochondria was measured in the absence of proton gradient. The basal mitochondrial respiration, which is the difference between the respiration rate of normal condition and the rate after adding complex I inhibitor rotenone (1 μM), was also determined.

Statistical analysis

Continuous variables were expressed as mean ± SD, and discrete variables in groups were expressed as frequency. Data were tested for normality using the one-sample

Kolmogorov-Smirnov test (2-tailed). Continuous variables between the two groups (maternally-related members and non-maternal members) were analyzed by both non-parametric methods (Mann-Whitney U test and two-sample Kolmogorov-Smirnov test) and parametric method (Independent samples t-test). Because of the limited sample size for each group (10 versus 16), non-parametric tests were preferred in this study. The parametric tests were also performed as they have more statistical power for the same number of observations under the assumption of normality or the assumption of homogeneity of variance. The Levene's test was applied to evaluate the equality of variances. Fisher's exact test (two-tailed) was used to compare the rates of the patient's gender, smoking and retinal microvascular structural changes (RMSC). Analysis of covariance (age, sex and BMI acting as simultaneous covariates) was used to compare biochemical test values, current visual acuity, ABPM parameters and echocardiographic parameters of the maternally-related members and non-maternal members. All statistical analyses were performed using SPSS 13.0 (SPSS Inc., Chicago, IL, USA), and $P<0.05$ was considered as statistically significant.

Result of normality tests for the related clinical data

We have employed one-sample Kolmogorov-Smirnov test (2-tailed) to test the normal distribution of the main variables. We evaluated the datasets for all members of family EH10 (n=26), maternal members (n=10) and non-maternal members (n=16), separately. All the main variables had a normal distribution ($P>0.05$) and we had included these results in supplementary Tables S4-S6.

References

[1] The sixth report of the Joint National Committee on prevention, detection, evaluation, and treatment of high blood pressure, Archives of international medicine. 157 (1997) 2413-2446.
[2] B. Gavish, I.Z. Ben-Dov, M. Bursztyn, Linear relationship between systolic and diastolic blood pressure monitored over 24 h: assessment and correlates, J. Hypertens. 26 (2008) 199-209.
[3] D.J. Sahn, A. DeMaria, J. Kisslo, A. Weyman, Recommendations regarding quantitation in M-mode echocardiography: results of a survey of echocardiographic measurements, Circulation. 58 (1978) 1072-1083.
[4] R.B. Devereux, D.R. Alonso, E.M. Lutas, G.J. Gottlieb, E. Campo, I. Sachs, N. Reichek, Echocardiographic assessment of left ventricular hypertrophy: comparison to necropsy findings, Am. J. Cardiol. 57 (1986) 450-458.
[5] H.G. Scheie, Evaluation of ophthalmoscopic changes of hypertension and arteriolar sclerosis, AMA. Arch. Ophthalmol. 49 (1953) 117-138.
[6] Y.G. Yao, H.J. Bandelt, N.S. Young, External contamination in single cell mtDNA analysis, PLoS One. 2 (2007) e681.
[7] H.W. Wang, X.Y. Jia, Y.L. Ji, Q.P. Kong, Q.J. Zhang, Y.G. Yao, Y.P. Zhang, Strikingly different penetrance of LHON in two Chinese families with primary mutation G11778A is independent of mtDNA haplogroup background and secondary mutation G13708A, Mutat. Res. 643 (2008) 48-53.
[8] R.M. Andrews, I. Kubacka, P.F. Chinnery, R.N. Lightowlers, D.M. Turnbull, N. Howell, Reanalysis and revision of the Cambridge reference sequence for human mitochondrial DNA, Nat. Genet. 23 (1999) 147.
[9] M. van Oven, M. Kayser, Updated comprehensive phylogenetic tree of global human mitochondrial DNA variation, Hum. Mutat. 30 (2009) E386-394.
[10] L. Fan, Y.G. Yao, MitoTool: a web server for the analysis and retrieval of human

mitochondrial DNA sequence variations, Mitochondrion. 11 (2011) 351-356.
[11] H.-J. Bandelt, A. Salas, R.W. Taylor, Y.-G. Yao, Exaggerated status of "novel" and "pathogenic" mtDNA sequence variants due to inadequate database searches, Hum. Mutat. 30 (2009) 191-196.
[12] Y. Ji, A.M. Zhang, X. Jia, Y.P. Zhang, X. Xiao, S. Li, X. Guo, H.J. Bandelt, Q. Zhang, Y.G. Yao, Mitochondrial DNA haplogroups M7b1'2 and M8a affect clinical expression of leber hereditary optic neuropathy in Chinese families with the m.11778G-->a mutation, Am. J. Hum. Genet. 83 (2008) 760-768.
[13] R. Bi, A.M. Zhang, W. Zhang, Q.P. Kong, B.L. Wu, X.H. Yang, D. Wang, Y. Zou, Y.P. Zhang, Y.G. Yao, The acquisition of an inheritable 50-bp deletion in the human mtDNA control region does not affect the mtDNA copy number in peripheral blood cells, Hum. Mutat. 31 (2010) 538-543.
[14] M. Simarro, A. Gimenez-Cassina, N. Kedersha, J.B. Lazaro, G.O. Adelmant, J.A. Marto, K. Rhee, S. Tisdale, N. Danial, C. Benarafa, A. Orduna, P. Anderson, Fast kinase domain-containing protein 3 is a mitochondrial protein essential for cellular respiration, Biochem. Biophys. Res. Commun. 401 (2010) 440-446.

Supplementary Tables

Table S1. Casual blood pressure of maternally-related members in family EH10

Generation (no. of individuals)	Age of onset (years)	SBP (mmHg)	DBP (mmHg)	PP (mmHg)
II (n=2)	49.0±1.4	180.0±0.0	90.0±0.0	90.0±0.0
III (n=4)	41.7±2.9	162.5±28.7	92.5±17.1	70.0±14.1
IV (n=5)	47.0±1.0	146.2±22.3	89.6±19.3	56.6±3.4
V (n=3)	NA	115.0±5.0	70.7±7.5	44.3±4.0

SBP – systolic blood pressure; DBP – diastolic blood pressure; PP – pulse pressure; NA – not available.

Table S2. Adjusted values (age, sex and BMI acting as simultaneous covariates) of clinical parameters for all 26 members in family EH10

Parameter	Maternal members (*n*=10)	Non-maternal members (*n*=16)	*F*-value	*P*-value
Biochemical values				
FBS, mmol/L	4.51±0.11	4.69±0.08	1.841	0.189
TC, mmol/L	4.51±0.20	4.70±0.16	0.462	0.549
TG, mmol/L	1.16±0.20	1.52±0.16	1.891	0.184
HDL, mmol/L	1.43±0.09	1.32±0.07	0.849	0.367
LDL, mmol/L	2.52±0.24	2.91±0.19	1.611	0.218
UA, μmol/L	262.98±30.19	260.76±23.67	0.003	0.955
BUN, mmol/L	5.44±0.25	5.77±0.20	1.049	0.317
Cr, μmol/L	102.17±4.72	97.27	0.646	0.430
Optical examinations				
Current visual acuity (OD)	0.74±0.12	0.66±0.10	0.255	0.619
Current visual acuity (OS)	0.79±0.11	0.68±0.09	0.580	0.455
ABPM parameters				
24hSBP, mmHg	110.87±1.71	102.39±1.34	14.779	0.001*
24hDBP, mmHg	64.37±1.57	68.83±1.23	4.820	0.040*
24hPP, mmHg	46.50±1.55	33.56±1.22	21.202	<0.001*
dSBP, mmHg	115.05±1.91	107.65±1.50	9.036	0.007*
dDBP, mmHg	67.38±1.64	71.51±1.29	3.800	0.065
dPP, mmHg	47.67±1.50	36.14±1.18	35.502	<0.001*
nSBP, mmHg	103.12±1.69	95.86±1.32	11.084	0.003*
nDBP, mmHg	60.00±1.81	64.56±1.42	3.806	0.065
nPP, mmHg	42.12±1.61	31.30±1.26	32.477	<0.001*
S-AASI	0.34±0.02	0.19±0.02	28.217	<0.001*
AASI	0.47±0.02	0.29±0.02	41.951	<0.001*
24hHR, bpm	75.57±2.66	75.27±2.08	0.008	0.930
Aorta's compliance				
PWV, m/sec	11.03±0.25	7.73±0.20	101.503	<0.001*
Echocardiographic				

parameters				
LV_d, mm	42.85±0.75	39.53±0.59	11.744	0.003*
LA, mm	32.53±1.04	28.66±0.82	8.271	0.009*
IVS, mm	9.74±0.29	8.36±0.23	13.950	0.001*
LVPW, mm	9.23±0.27	8.01±0.21	12.450	0.002*
LVMI, g/m^2	97.31±6.02	64.87±4.72	17.399	<0.001*
EF, %	63.76±0.86	63.84±0.67	0.005	0.946
E, m/s	0.90±0.04	0.93±0.03	0.533	0.473
A, m/s	0.81±0.04	0.73±0.03	3.313	0.083
E/A	1.13±0.05	1.29±0.04	5.677	0.027*

Data are expressed as adjusted mean ± SEM. Data are adjusted for age, sex and BMI differences between two groups.
FBS: fasting blood sugar; TC: total cholesterol; TG: triglyceride; HDL: high-density lipoprotein; LDL: low-density lipoprotein; UA: urea acid; BUN: blood urea nitrogen; Cr: creatine; OD: right eye; OS: left eye; ABPM: ambulatory blood pressure monitoring; 24hSBP: 24 hour systolic blood pressure; 24hDBP: 24 hour diastolic blood pressure; 24hPP: 24 hour pulse pressure; dSBP: day systolic blood pressure; dDBP: day diastolic blood pressure; dPP: day pulse pressure; nSBP: night systolic blood pressure; nDBP: night diastolic blood pressure; nPP: night pulse pressure; S-AASI: symmetric ambulatory arterial stiffness index; AASI: ambulatory arterial stiffness index; 24hHR: 24 hour heart rate; PWV: pulse wave velocity; LV_d: left ventricular diastolic diameter; LA: left atrium diameter; IVS: interventricular septum thickness; LVPW: left ventricular posterior wall thickness; LVMI: left ventricular mass index; EF: ejection fraction; E: early mitral inflow velocity; A: Late mitral inflow velocity.
* A *P* value <0.05 was marked by a star.

Table S3. Echocardiographic data of 26 members in family EH10

Subject	Gender	Age at test (year)	LVd (mm)	LA (mm)	IVS (mm)	LVPW (mm)	LVMI (g/m^2)	EF (%)	E (m/s)	A (m/s)	E/A
Maternal members of proband											
III:5	F	73	50.00	46.00	10.60	10.60	148.06	65.3	1.01	1.04	0.97
III:7	M	69	44.00	33.00	10.10	9.00	89.99	66.3	0.94	0.69	1.36
IV:1	F	52	42.00	33.00	11.00	10.80	121.02	65.2	0.83	0.92	0.90
IV:3	F	50	41.00	32.00	10.20	10.00	106.88	61.2	0.87	0.70	1.24
IV:5	M	47	46.00	35.00	11.00	10.20	121.98	60.1	0.88	0.95	0.93
IV:9	F	44	45.00	33.00	10.00	10.20	117.84	61.8	0.72	0.86	0.84
IV:11	M	42	44.00	32.00	11.30	9.20	97.72	64.8	0.88	0.69	1.28
V:1	F	27	38.00	21.00	7.40	7.20	57.12	69.5	0.83	0.61	1.36
V:2	F	19	36.00	26.00	7.20	7.00	45.90	62.4	1.01	0.84	1.20
V:4	F	24	37.00	29.00	7.10	7.20	49.61	63.8	1.00	0.78	1.28
Non-maternal members of proband											
III:2	M	81	45.00	32.00	9.30	9.10	87.11	61.2	0.84	0.65	1.29
III:4	F	68	40.00	31.50	9.00	9.00	83.07	64.8	1.01	0.84	1.20
III:6	M	76	42.00	30.80	9.10	8.90	74.81	60.9	0.87	0.69	1.26
III:8	F	65	40.00	30.50	8.50	8.60	69.58	66.2	0.94	0.85	1.11
IV:2	M	55	38.00	29.40	8.00	7.40	49.61	64.4	0.90	0.80	1.13
IV:4	M	53	40.00	31.20	8.20	7.60	57.72	60.9	1.16	0.74	1.57
IV:6	F	45	39.00	26.00	8.00	7.20	57.86	62.7	0.75	0.62	1.21
IV:7	M	48	39.00	30.00	8.40	7.80	56.45	63.8	1.13	0.90	1.26
IV:8	F	52	40.00	29.00	9.10	8.40	74.51	69.9	0.92	0.68	1.35
IV:10	M	46	42.00	31.00	9.00	9.00	76.45	61.9	0.88	0.69	1.28
IV:12	F	40	38.00	27.00	8.20	7.80	64.71	64.9	0.76	0.62	1.23
IV:13	F	44	37.00	26.50	8.00	7.50	54.41	67.6	0.81	0.73	1.11
IV:14	M	42	40.00	27.40	8.30	7.60	61.92	60.1	0.92	0.82	1.12
IV:15	M	41	41.00	28.20	8.00	7.40	57.34	61.8	1.10	0.75	1.47
V:3	F	23	37.00	26.40	8.10	7.80	61.81	66.3	1.08	0.71	1.52
V:5	F	18	40.00	27.00	8.00	7.90	67.60	61.2	0.84	0.61	1.38

LVd: left ventricular diastolic diameter; LA: left atrium diameter; IVS: interventricular septum thickness; LVPW: left ventricular posterior wall thickness; LVMI: left ventricular mass index; EF: ejection fraction; E: early mitral inflow velocity; A: Late mitral inflow velocity.

Table S4. Tests for normality for main continuous variables of all 26 members in family EH10

Parameter	Members of EH10 (*n*=26)	D-value	Kolmogorov -Smirnov Z	*P*-value
General information				
Age, years	47.85±17.15	0.131	0.670	0.761
BMI, kg/m^2	22.12±2.29	0.148	0.753	0.622
WHR	0.80±0.08	0.103	0.527	0.944
Biochemical values				
FBS, mmol/L	4.62±0.33	0.168	0.854	0.459
TC, mmol/L	4.63±0.59	0.112	0.573	0.898
TG, mmol/L	1.38±0.60	0.227	1.157	0.137
HDL, mmol/L	1.36±0.28	0.111	0.569	0.903
LDL, mmol/L	2.76±0.70	0.223	1.14	0.151
UA, μmol/L	261.62±91.47	0.179	0.913	0.376
BUN, mmol/L	5.64±0.79	0.103	0.525	0.946
Cr, μmol/L	99.15±15.85	0.133	0.676	0.751
Optical examinations				
Current visual acuity (OD)	0.69±0.39	0.119	0.607	0.854
Current visual acuity (OS)	0.72±0.34	0.141	0.720	0.678
ABPM parameters				
24hSBP, mmHg	105.65±9.90	0.182	0.930	0.353
24hDBP, mmHg	67.11±5.85	0.119	0.606	0.857
24hPP, mmHg	38.54±8.90	0.222	1.135	0.152
dSBP, mmHg	110.50±9.81	0.176	0.898	0.395
dDBP, mmHg	69.92±5.89	0.141	0.720	0.677
dPP, mmHg	40.58±8.26	0.182	0.926	0.357
nSBP, mmHg	98.65±8.98	0.183	0.932	0.350
nDBP, mmHg	62.81±6.18	0.141	0.721	0.676
nPP, mmHg	35.85±8.44	0.138	0.703	0.706
S-AASI	0.24±0.11	0.173	0.883	0.417
AASI	0.36±0.13	0.137	0.701	0.710
24hHR, bpm	75.38±9.02	0.098	0.502	0.963

Aorta's compliance				
PWV, m/sec	9.00±2.04	0.153	0.781	0.576
Echocardiographic parameters				
LV_d, mm	40.81±3.30	0.174	0.885	0.413
LA, mm	30.15±4.44	0.184	0.937	0.344
IVS, mm	8.89±1.21	0.164	0.836	0.487
LVPW, mm	8.48±1.16	0.190	0.971	0.303
LVMI, g/m^2	77.35±26.82	0.167	0.853	0.461
EF, %	63.81±2.75	0.140	0.716	0.684
E, m/s	0.92±0.12	0.130	0.662	0.774
A, m/s	0.76±0.11	0.133	0.679	0.745
E/A	1.23±0.18	0.137	0.699	0.712

BMI: body mass index; WHR: waist hip ratio; FBS: fasting blood sugar; TC: total cholesterol; TG: triglyceride; HDL: high-density lipoprotein; LDL: low-density lipoprotein; UA: urea acid; BUN: blood urea nitrogen; Cr: creatine; OD: right eye; OS: left eye; RMSC: retinal microvascular structural changes; ABPM: ambulatory blood pressure monitoring; 24hSBP: 24 hour systolic blood pressure; 24hDBP: 24 hour diastolic blood pressure; 24hPP: 24 hour pulse pressure; dSBP: day systolic blood pressure; dDBP: day diastolic blood pressure; dPP: day pulse pressure; nSBP: night systolic blood pressure; nDBP: night diastolic blood pressure; nPP: night pulse pressure; S-AASI: symmetric ambulatory arterial stiffness index; AASI: ambulatory arterial stiffness index; 24hHR: 24 hour heart rate; PWV: pulse wave velocity; LV_d: left ventricular diastolic diameter; LA: left atrium diameter; IVS: interventricular septum thickness; LVPW: left ventricular posterior wall thickness; LVMI: left ventricular mass index; EF: ejection fraction; E: early mitral inflow velocity; A: Late mitral inflow velocity.

Table S5. Tests for normality for main continuous variables of maternally-related members in family EH10

Parameter	Maternal members (*n*=10)	D-value	Kolmogorov -Smirnov Z	*P*-value
General information				
Age, years	44.70±17.91	0.142	0.448	0.988
BMI, kg/m^2	21.59±2.54	0.190	0.602	0.861
WHR	0.79±0.09	0.227	0.717	0.683
Biochemical values				
FBS, mmol/L	4.49±0.38	0.190	0.601	0.863
TC, mmol/L	4.54±0.32	0.250	0.790	0.561
TG, mmol/L	1.18±0.23	0.238	0.752	0.624
HDL, mmol/L	1.43±0.23	0.169	0.534	0.938
LDL, mmol/L	2.53±0.47	0.152	0.481	0.975
UA, μmol/L	255.50±71.76	0.241	0.764	0.604
BUN, mmol/L	5.40±0.94	0.200	0.632	0.819
Cr, μmol/L	101.20±16.88	0.221	0.699	0.713
Optical examinations				
Current visual acuity (OD)	0.72±0.40	0.183	0.580	0.889
Current visual acuity (OS)	0.78±0.33	0.176	0.557	0.916
ABPM parameters				
24hSBP, mmHg	110.30±12.07	0.208	0.657	0.781
24hDBP, mmHg	64.60±4.17	0.232	0.732	0.657
24hPP, mmHg	45.70±10.12	0.146	0.462	0.983
dSBP, mmHg	114.70±12.24	0.179	0.567	0.905
dDBP, mmHg	67.50±4.45	0.245	0.774	0.587
dPP, mmHg	47.20±9.19	0.152	0.481	0.975
nSBP, mmHg	102.70±11.00	0.186	0.587	0.881
nDBP, mmHg	60.20±3.97	0.280	0.885	0.414
nPP, mmHg	42.50±9.07	0.209	0.660	0.777
S-AASI	0.32±0.13	0.131	0.414	0.995
AASI	0.46±0.14	0.147	0.465	0.982

24hHR, bpm	76.20±7.61	0.163	0.514	0.954
Aorta's compliance				
PWV, m/sec	10.76±1.97	0.151	0.479	0.976
Echocardiographic parameters				
LV_d, mm	42.30±4.40	0.150	0.476	0.977
LA, mm	32.00±6.45	0.238	0.754	0.621
IVS, mm	9.59±1.68	0.296	0.937	0.344
LVPW, mm	9.14±1.49	0.218	0.689	0.729
LVMI, g/m^2	95.61±34.66	0.167	0.527	0.944
EF, %	64.04±2.78	0.125	0.397	0.998
E, m/s	0.90±0.09	0.172	0.543	0.930
A, m/s	0.81±0.14	0.184	0.582	0.887
E/A	1.14±0.20	0.224	0.708	0.698

BMI: body mass index; WHR: waist hip ratio; FBS: fasting blood sugar; TC: total cholesterol; TG: triglyceride; HDL: high-density lipoprotein; LDL: low-density lipoprotein; UA: urea acid; BUN: blood urea nitrogen; Cr: creatine; OD: right eye; OS: left eye; RMSC: retinal microvascular structural changes; ABPM: ambulatory blood pressure monitoring; 24hSBP: 24 hour systolic blood pressure; 24hDBP: 24 hour diastolic blood pressure; 24hPP: 24 hour pulse pressure; dSBP: day systolic blood pressure; dDBP: day diastolic blood pressure; dPP: day pulse pressure; nSBP: night systolic blood pressure; nDBP: night diastolic blood pressure; nPP: night pulse pressure; S-AASI: symmetric ambulatory arterial stiffness index; AASI: ambulatory arterial stiffness index; 24hHR: 24 hour heart rate; PWV: pulse wave velocity; LV_d: left ventricular diastolic diameter; LA: left atrium diameter; IVS: interventricular septum thickness; LVPW: left ventricular posterior wall thickness; LVMI: left ventricular mass index; EF: ejection fraction; E: early mitral inflow velocity; A: Late mitral inflow velocity.

Table S6. Tests for normality for main continuous variables of non-maternal members in family EH10

Parameter	Non-maternal members (*n*=16)	D-value	Kolmogorov-Smirnov Z	*P*-value
General information				
Age, years	49.81±16.94	0.156	0.625	0.830
BMI, kg/m^2	22.45±2.13	0.129	0.518	0.951
WHR	0.82±0.07	0.108	0.432	0.992
Biochemical values				
FBS, mmol/L	4.70±0.28	0.174	0.694	0.721
TC, mmol/L	4.68±0.71	0.188	0.753	0.622
TG, mmol/L	1.51±0.72	0.202	0.808	0.532
HDL, mmol/L	1.32±0.31	0.160	0.640	0.807
LDL, mmol/L	2.90±0.79	0.240	0.959	0.317
UA, μmol/L	265.44±103.99	0.165	0.660	0.777
BUN, mmol/L	5.79±0.66	0.167	0.667	0.765
Cr, μmol/L	97.88±15.60	0.145	0.583	0.886
Optical examinations				
Current visual acuity (OD)	0.67±0.40	0.129	0.517	0.952
Current visual acuity (OS)	0.69±0.35	0.126	0.504	0.961
ABPM parameters				
24hSBP, mmHg	102.75±7.26	0.166	0.665	0.769
24hDBP, mmHg	68.69±6.31	0.145	0.579	0.890
24hPP, mmHg	34.06±3.86	0.167	0.666	0.767
dSBP, mmHg	107.88±7.17	0.134	0.534	0.938
dDBP, mmHg	71.44±6.29	0.105	0.420	0.995
dPP, mmHg	36.44±3.95	0.142	0.568	0.903
nSBP, mmHg	96.13±6.65	0.139	0.556	0.917
nDBP, mmHg	64.44±6.85	0.167	0.667	0.766
nPP, mmHg	31.69±4.63	0.156	0.623	0.832
S-AASI	0.20±0.06	0.151	0.604	0.858

AASI	0.30±0.07	0.136	0.544	0.929
24hHR, bpm	74.88±10.00	0.90	0.361	0.999
Aorta's compliance				
PWV, m/sec	7.89±1.10	0.142	0.566	0.905
Echocardiographic parameters				
LV_d, mm	39.88±2.03	0.225	0.902	0.390
LA, mm	28.99±2.06	0.155	0.620	0.837
IVS, mm	8.45±0.48	0.199	0.795	0.553
LVPW, mm	8.06±0.66	0.222	0.889	0.408
LVMI, g/m^2	65.94±10.81	0.148	0.590	0.877
EF, %	63.66±2.81	0.172	0.688	0.731
E, m/s	0.93±0.13	0.162	0.650	0.793
A, m/s	0.73±0.09	0.115	0.461	0.984
E/A	1.28±0.14	0.162	0.647	0.797

BMI: body mass index; WHR: waist hip ratio; FBS: fasting blood sugar; TC: total cholesterol; TG: triglyceride; HDL: high-density lipoprotein; LDL: low-density lipoprotein; UA: urea acid; BUN: blood urea nitrogen; Cr: creatine; OD: right eye; OS: left eye; RMSC: retinal microvascular structural changes; ABPM: ambulatory blood pressure monitoring; 24hSBP: 24 hour systolic blood pressure; 24hDBP: 24 hour diastolic blood pressure; 24hPP: 24 hour pulse pressure; dSBP: day systolic blood pressure; dDBP: day diastolic blood pressure; dPP: day pulse pressure; nSBP: night systolic blood pressure; nDBP: night diastolic blood pressure; nPP: night pulse pressure; S-AASI: symmetric ambulatory arterial stiffness index; AASI: ambulatory arterial stiffness index; 24hHR: 24 hour heart rate; PWV: pulse wave velocity; LV_d: left ventricular diastolic diameter; LA: left atrium diameter; IVS: interventricular septum thickness; LVPW: left ventricular posterior wall thickness; LVMI: left ventricular mass index; EF: ejection fraction; E: early mitral inflow velocity; A: Late mitral inflow velocity.

Table S7. Mitochondrial function in lymphoblastoid cell lines derived from patients carrying mutation m.14484T>C and healthy subjects

Parameter	Control cell line				Mutant cell line				*P*-value [b]
	C1	C2	C3	Group mean	III:5	IV:1	V:1	Group mean	
ATP-coupled respiration [a]	0.53±0.22	0.36±0.17	0.50±0.04	0.46±0.09	0.12±0.12	0.11±0.05	0.12±0.01	0.11±0.01	0.003
Basal mitochondrial respiration [a]	0.65±0.18	0.57±0.28	0.68±0.09	0.63±0.05	0.31±0.17	0.12±0.05	0.14±0.04	0.19±0.10	0.003
Maximal respiratory capacity [a]	0.68±0.23	0.72±0.42	0.81±0.18	0.74±0.06	0.39±0.23	0.16±0.16	0.16±0.13	0.24±0.13	0.004
ΔΨm	49.06±2.06	65.61±14.03	67.23±9.96	60.63±10.06	6.47±3.16	12.69±9.38	2.77±1.09	7.31±5.01	0.001
ROS	17.82±14.58	14.99±1.03	7.84±4.97	13.55±5.15	47.44±25.27	26.95±11.26	23.40±19.64	32.60±12.98	0.077
Mitochondrial mass	61.10±13.28	30.37±1.44	44.44±4.64	45.30±15.38	49.72±13.81	68.94±5.25	68.04±9.37	62.23±10.84	0.194
ATP	19966±4501	17181±2338	20011±4584	19053±1621	19267±5420	15772±2763	19570±4690	18203±2111	0.610

[a] Value for each oxygen consumption rate parameter (including ATP-coupled respiration, basal mitochondrial respiration and maximal respiratory capacity) was normalized to its initial value before adding inhibitors, which was arbitrarily set to 1.

[b] Two-tailed, unpaired *t*-test

Supplementary Figures and Figure Legends

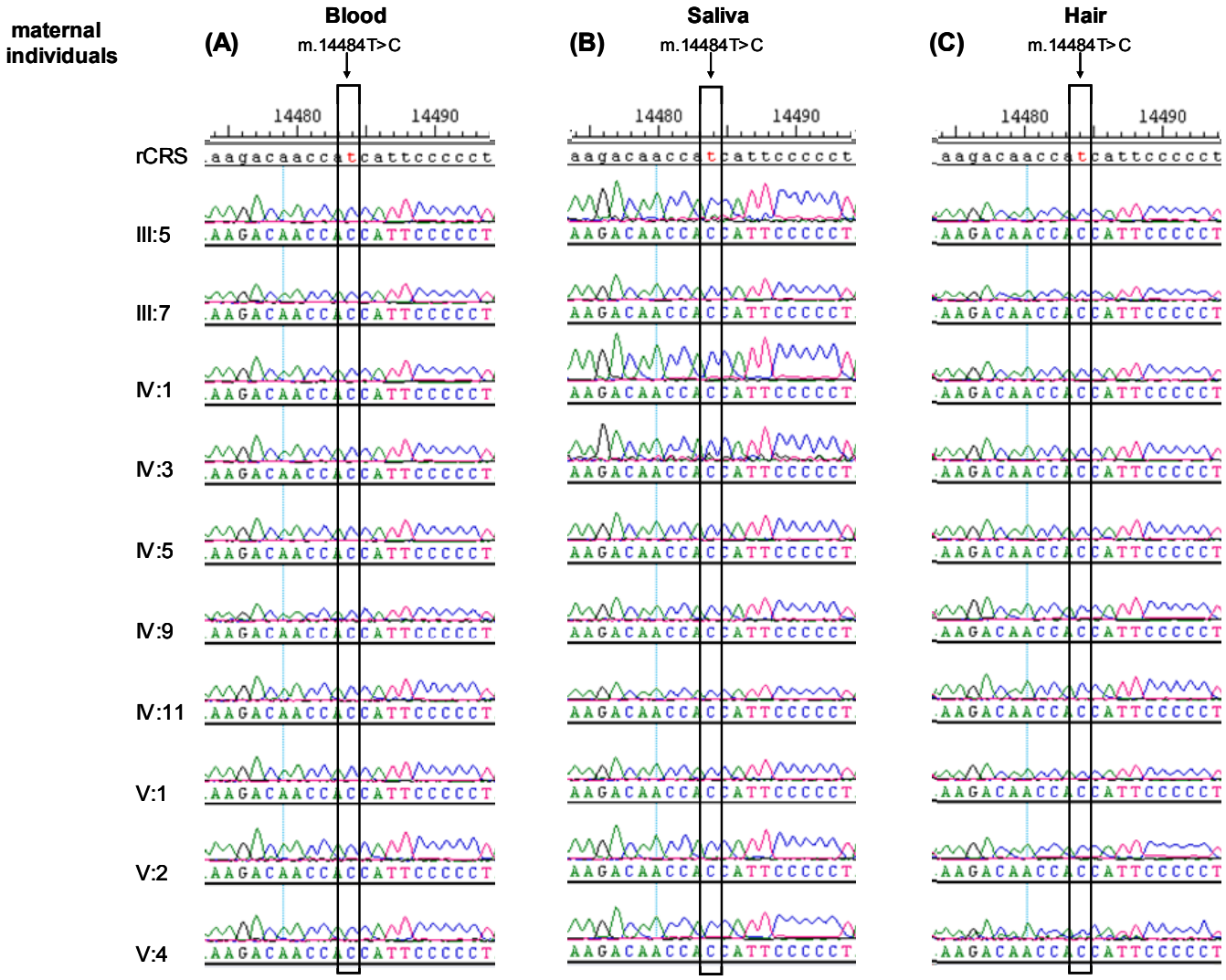

Figure S1. Sequence chromatograms showing the presence of m.14484T>C in different tissues from maternally-related members of proband IV:1 in family EH10.
Mutation m.14484T>C was homoplasmic in all three tissues (whole blood, saliva and hair root) from the proband and her maternally-related relatives. Arrow indicates position 14484. The individual information is shown in Figure 1.

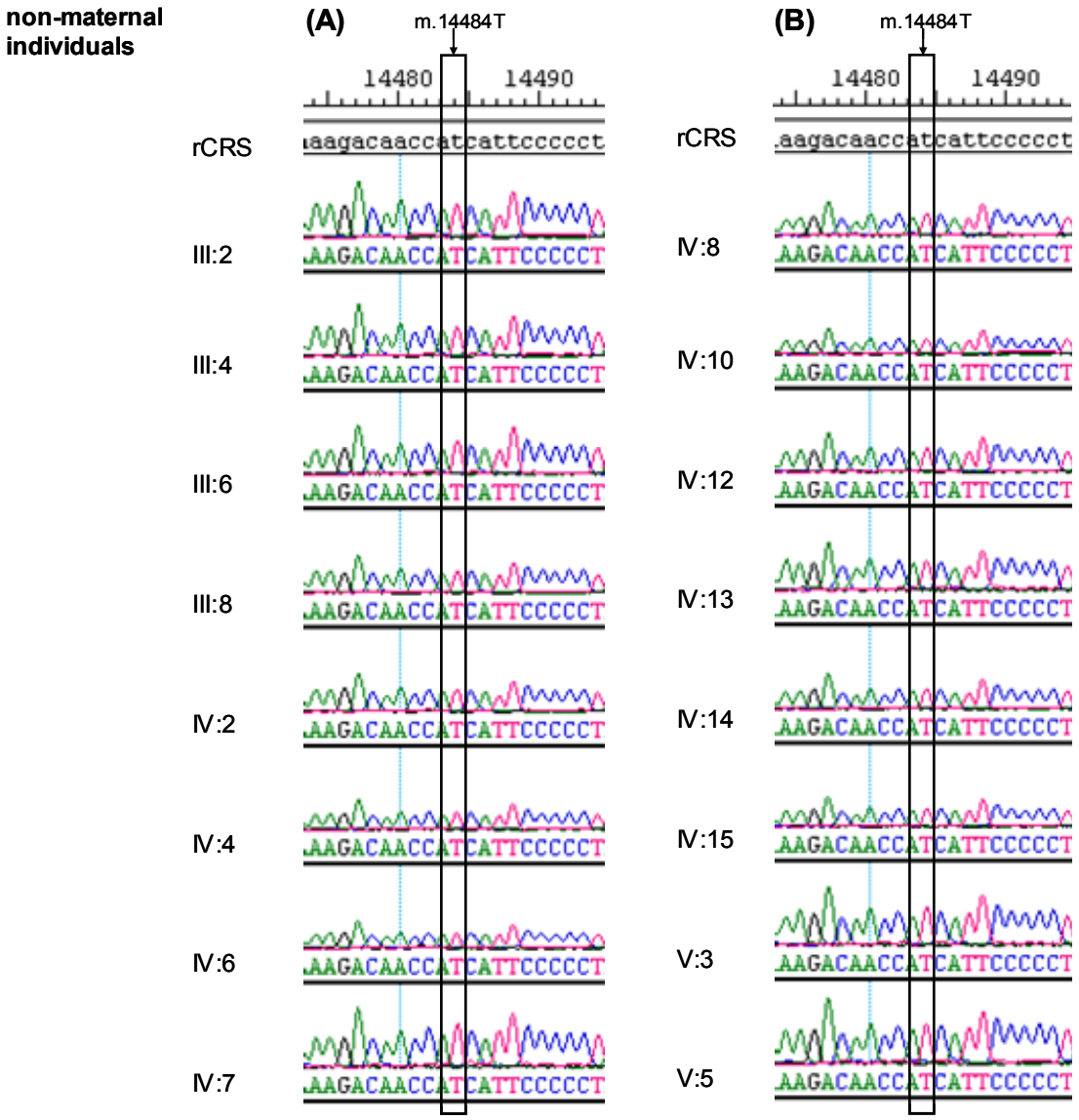

Figure S2. Sequence chromatograms showing the absence of m.14484T>C in blood samples from non-maternal individuals of proband IV:1 in family EH10.
Arrow indicates position 14484. The individual information is shown in Figure 1.

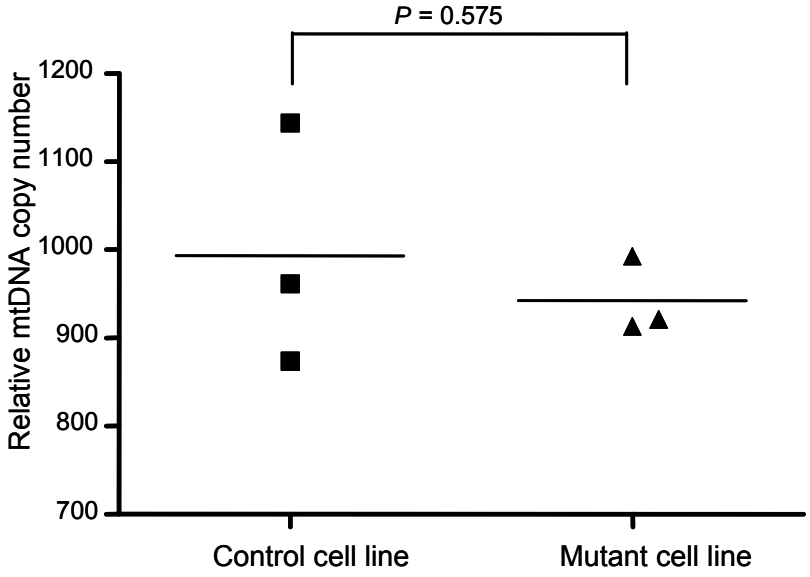

Figure S3. Relative mtDNA copy number in lymphoblastoid cell lines established from three maternally-related members in family EH10 and three unrelated healthy donors.

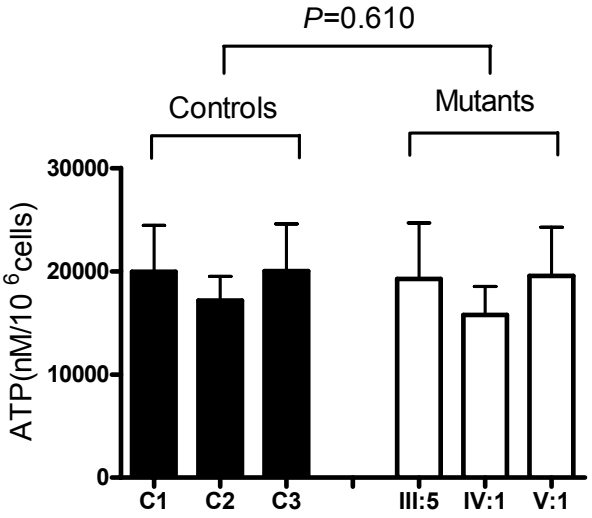

Figure S4. Cellular ATP levels in lymphoblastoid cell lines established from three maternally-related members in family EH10 and three unrelated healthy donors.
Bars represent mean ± SD of triplicate experiments.