ORIGINAL RESEARCH

Mitochondrial tRNA Variants in Chinese Subjects With Coronary Heart Disease

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***Background*—**Coronary heart disease is the leading cause of death worldwide. Mitochondrial genetic determinants for the development of this disorder remain less explored.

***Methods and Results*—**We performed a clinical and genetic evaluation and mutational screening of 22 mitochondrial tRNA genes in a cohort of 80 genetically unrelated Han Chinese subjects and 125 members of 4 families with coronary heart disease and 512 Chinese control subjects. This analysis identified 16 nucleotide changes among 9 tRNA genes. Of these, the T5592C mutation creates a highly conservative base pairing (5G-68C) on the acceptor stem of $tRNA^{Gln}$, whereas the G15927A mutation destabilizes a highly conserved base pairing (28C-42G) in the anticodon stem of $tRNA^{Thr}$. However, the other tRNA variants were polymorphisms. The pedigrees of BJH24 carrying the T5592C mutation, BJH15, and BJH45 harboring the G15927A mutation exhibited maternal transmission of coronary heart disease. Sequence analysis of their mitochondrial genomes revealed the presence of T5592C or G15927A mutation but the absence of other functionally significant mutations in all matrilineal relatives of these families.

***Conclusions*—**Our previous observations showed that altered structures of tRNAs by these mtDNA mutations caused mitochondrial dysfunction. These may be the first evidence that mtDNA mutations increase the risk of coronary heart disease. Our findings may provide new insights into the pathophysiology of this disorder. **(*J Am Heart Assoc*. 2014;3:e000437 doi: 10.1161/JAHA.113.000437)**

Key Words: coronary heart disease • maternal transmission • mitochondrial tRNA • mutation

Coronary heart disease is a leading cause of death worldwide. In particular, coronary heart disease (CHD) annually results in 502 000 deaths in the United States and >700 000 deaths in China.[1,2] CHD is a common complex disorder that can be caused by single gene or multifactorial conditions resulting from interactions between environmental and inherited risk factors.[3–5] Efforts to identify genetic determinants of CHD have been directed primarily at nuclear genes.[6] Genome-wide association studies in the population of European and Asian ancestries have identified several genetic loci that are associated with risk of CHD.[7–9] However, the role of mitochondrial genetic defects in the development of coronary heart disease remains poorly understood.[10,11] The human mitochondrial genome encodes 13 peptides for the oxidative phosphorylation system, 2 rRNAs, and 22 tRNAs required for mitochondrial protein synthesis.[12] Among these tRNAs, $tRNA^{Glu}$, $tRNA^{Ala}$, $tRNA^{Asn}$, $tRNA^{Cys}$, $tRNA^{Tyr}$, $tRNA^{Ser(UCN)}$, $tRNA^{Gln}$, and $tRNA^{Pro}$ reside in the cytosine-rich light strand; the remaining $tRNA^{Phe}$, $tRNA^{Val}$, $tRNA^{Leu(UUR)}$, $tRNA^{Leu(CUN)}$, $tRNA^{Ile}$, $tRNA^{Met}$, $tRNA^{Ser(AGY)}$, $tRNA^{Trp}$, $tRNA^{Asp}$, $tRNA^{Lys}$, $tRNA^{Gly}$, $tRNA^{Arg}$, $tRNA^{His}$, and $tRNA^{Thr}$ are in the guanine-rich heavy strand.[12,13] Mitochondrial tRNA genes are the hot spots for mutations associated with cardiovascular disorders such as hypertension.[14–17] These hypertension-associated tRNA mutations included the T4291C and A4263G mutations in the $tRNA^{Ile}$ gene, the A4435G mutation in the $tRNA^{Met}$ gene, and the A4401G mutation at the junction of the $tRNA^{Met}$ and $tRNA^{Gln}$ genes.[18–20]

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It is anticipated that mutations in mitochondrial tRNA genes are associated with coronary heart disease. To investigate the role of mitochondrial genetic defects in the development of coronary heart disease, we carried out a systematic and extended mutational screening of 22 tRNA genes in a cohort of 80 Han Chinese subjects with coronary heart disease. Mutational analysis of these tRNA genes in these subjects identified 16 nucleotide changes in 9 tRNA genes. These tRNA variants were further evaluated by phylogenetic analysis, structure–function relation, and allelic frequency of these variants in the Han Chinese controls from the same region.

Methods

Subjects

A total of 80 genetically unrelated Chinese subjects with coronary heart disease, aged 33 to 79 years old from Beijing, along with some of their family members, were enrolled in this study under an institutional review board–approved protocol of informed consent at the Zhejiang University Institutional Review Board and Ethics Committee of Beijing Anzhen Hospital, China. Members of these families were interviewed and evaluated to identify both personal and medical histories of CHD and other clinical abnormalities. The 512 control DNA samples were obtained from a panel of unaffected Han Chinese individuals from the same area.

Subjects underwent a physical examination and laboratory assessment of cardiovascular disease risk factors. A heart function evaluation and measurement of systolic and diastolic blood pressure of subjects were performed as detailed elsewhere.[20] All patients underwent a heart function evaluation by electrocardiography. Signals from the first 10 seconds of the conventional electrocardiography recording were analyzed automatically in software to quantify all major intervals, axes, and voltages as well as ST-segment levels. The initial candidate criteria used for defining these strictly conventional 12-lead electrocardiograms were as follows. Eligible patients had ≥10 minutes of ischemic symptoms at rest and presented with 1 of the following: elevated markers of myonecrosis, ≥0.1 mV of ST depression, or diabetes mellitus. Patients were referred for angiography for suspected myocardial ischemia such as myocardial infarction or unstable angina and myocardial infarction as symptoms and increased troponin I, with or without ST elevation on the electrocardiogram. Coronary angiography was performed by the Judkins technique, and images of the coronary tree were obtained in routine, standardized projections. The angiograms were assessed by ≥2 cardiologists. Coronary angiograms were visually evaluated by 3 independent experienced observers according to the clinical review process. Localization and percentage of luminal diameter reduction were documented for any coronary artery with a stenosis. Significant coronary artery stenosis was defined as luminal diameter reduction of 50%. Patients without angiographic lesions were considered the patients without CHD.[21]

Hypertension, hyperlipidemia, diabetes mellitus, cigarette smoking, and family history for CHD were considered risk factors. Diabetes mellitus was defined as hyperglycemia requiring antidiabetic drugs or fasting blood sugar >126 g/dL. Patients reporting cigarette use during the year prior to examination were considered smokers. Hyperlipidemia was defined as plasma low-density lipoprotein cholesterol >130 mg/dL or total cholesterol >200 mg/dL or using lipid-lowering drugs at the time of investigation. Overweight for Chinese subjects was defined as body mass index >24.

Mutational Analysis of Mitochondrial Genome

Genomic DNA was isolated from the whole blood of participants using Puregene DNA Isolation Kits (Gentra Systems, Minneapolis, MN). The fragments spanning all 22 of the tRNA genes of 80 subjects with CHD and 512 control subjects were PCR-amplified by use of sets of the light-strand and the heavy-strand oligonucleotide primers (Table 1). The entire mitochondrial genomes of 3 probands (BJH24-II-2, BJH15-III-2, and BJH45-III-7) were PCR-amplified in 24 overlapping fragments by use of sets of the light-strand and the heavy-strand oligonucleotide primers, as described elsewhere.[22] Each fragment was purified and subsequently analyzed by direct sequencing in an ABI 3700 automated DNA sequencer using a Big Dye Terminator Cycle sequencing reaction kit. The resultant sequence data were compared with the updated consensus Cambridge sequence (GenBank accession number: NC_012920).[12]

Structural Analysis

The published secondary structures for the tRNAs were used to define the stem-and-loop structure.[17,23]

Phylogenetic Analysis

A total of 17 vertebrates' mitochondrial DNA sequences were used in the interspecific analysis. These included *Bos taurus*, *Cebus albifrons*, *Gorilla gorilla*, *Homo sapiens*, *Hylobates lar*, *Lemur catta*, *Macaca mulatta*, *Macaca sylvanus*, *Mus musculus*, *Nycticebus coucang*, *Pan paniscus*, *Pan troglodytes*, *Papio hamadryas*, *Pongo abelii*, *Pongo pygmaeus*,

Table 1. Oligonucleotide Primers for Amplification of 22 Human Mitochondrial tRNAs

Locus	Starting	Ending	Length (bp)	Number of Variants	Primer[22]	Primer Sequence 5′-3′
$tRNA^{Phe}$	577	647	71	5	1F/1R	CTCCTCAAAGCAATACACTG/TGCTAAATCCACCTTCGACC
$tRNA^{Val}$	1602	1670	69	5	2F/2R	CGATCAACCTCACCACCTCT/TGGACAACCAGCTATCACCA
$tRNA^{Leu(UUR)}$	3230	3304	75	6	4F/4R	AAATCTTACCCCGCCTGTTT/AGGAATGCCATTGCGATTAG
$tRNA^{Ile}$	4263	4331	69	10	6F/6R	TGGCTCCTTTAACCTCTCCA/AAGGATTATGGATGCGGTTG
$tRNA^{Gln}$	4329	4400	72	12	6F/6R	As above
$tRNA^{Met}$	4402	4469	68	6	6F/6R	As above
$tRNA^{Trp}$	5512	5579	68	8	7F/8R	ACTAATTAATCCCCTGGCCC/ACCTAGAAGGTTGCCTGGCT
$tRNA^{Ala}$	5587	5655	69	6	8F/8R	CTAACCGGCTTTTTGCCC/ACCTAGAAGGTTGCCTGGCT
$tRNA^{Asn}$	5657	5729	73	5	8F/8R	As above
$tRNA^{Cys}$	5761	5826	66	15	8F/8R	As above
$tRNA^{Tyr}$	5826	5891	66	4	8F/8R	As above
$tRNA^{Ser(UCN)}$	7446	7514	69	3	11F/11R	ACGCCAAAATCCATTTCACT/CGGGAATTGCATCTGTTTTT
$tRNA^{Asp}$	7518	7585	68	6	11F/11R	As above
$tRNA^{Lys}$	8295	8364	70	7	12F/12R	ACGAGTACACCGACTACGGC/TGGGTGGTTGGTGTAAATGA
$tRNA^{Gly}$	9991	10058	68	10	14F/15R	CCCACCAATCACATGCCTAT/AATTAGGCTGTGGGTGGTTG
$tRNA^{Arg}$	10405	10469	65	5	15F/15R	TCTCCATCTATTGATGAGGGTCT/AATTAGGCTGTGGGTGGTTG
$tRNA^{His}$	12138	12206	69	6	18F/18R	TATCACTCTCCTACTTACAG/AGAAGGTTATAATTCCTACG
$tRNA^{Ser(AGY)}$	12207	12265	59	11	18F/18R	As above
$tRNA^{Leu(CUN)}$	12266	12336	71	5	18F/18R	As above
$tRNA^{Glu}$	14674	14742	69	5	21F/21R	GCATAATTAAACTTTACTTC/AGAATATTGAGGCGCCATTG
$tRNA^{Thr}$	15888	15953	66	27	22F/23R	TGAAACTTCGGCTCACTCCT/GAGTGGTTAATAGGGTGATAG
$tRNA^{Pro}$	15956	16023	68	6	22F/23R	TGAAACTTCGGCTCACTCCT/GAGTGGTTAATAGGGTGATAG

Tarsius bancanus, and *Xenopus laevis* (Genbank; Table 2). The conservation index (CI) was calculated by comparing the human nucleotide variants with 16 other vertebrates. The CI was then defined as the percentage of species from the list of 17 different vertebrates that had the wild-type nucleotide at that position.

Statistics Analysis

Statistical analyses were performed using the SSPS statistical package, version 16.0, and statistical significance was established at $P<0.05$. We performed Fisher's exact test to evaluate the difference in mitochondrial tRNA mutations between CHD patients and controls.

Results

Study Samples

The patients in the study samples suffering from coronary heart disease alone or with other clinical phenotypes including hypertension, diabetes mellitus, and cerebrovascular disease, consisted of 57 men and 23 women. Clinical data for these 80 subjects are summarized in Table 3. All participants were Han Chinese from the Beijing area. Of these, 22 subjects only exhibited coronary heart disease. In addition to coronary heart disease, 40 subjects suffered from hypertension, 5 individuals had diabetes mellitus, 9 subjects exhibited hypertension and diabetes mellitus, 2 individuals had diabetes mellitus and cerebrovascular disease, 1 subject exhibited hypertension and cerebrovascular disease, and 1 subject suffered from hypertension, diabetes mellitus, and cerebrovascular disease. The age at onset of coronary heart disease in all participants ranged from 33 to 79 years, with a median age of 61 years. The age at onset of hypertension in 51 subjects varied from 15 to 77 years, with a median age of 48.8 years, whereas the age at onset of diabetes mellitus in 17 subjects ranged from 38 to 71 years, with an average age of 52.8 years. Body mass index of all participants ranged from 18.03 to 31.64, with an average of 25.39. Forty-two of 80 subjects were smokers. A total of 512 unaffected Han Chinese subjects were obtained from the same area. The age

Table 2. mtDNA Sequence of 17 Vertebrate Species

Species Name	GenBank Accession Number
Homo sapiens	NC_012920
Cebus albifron	NC_002763
Gorilla gorilla	NC_011120
Hylobates lar	NC_002082
Lemur catta	NC_004025
Macaca mulatta	NC_005943
Macaca sylvanus	NC_002764
Nycticebus coucang	NC_002765
Pan paniscus	NC_001644
Pan troglodytes	NC_001643
Papio hamadryas	NC_001992
Pongo pygmaeus	NC_001646
Pongo pygmaeus abelii	NC_002083
Tarsius bancanus	NC_002811
Mus musculus	NC_006914.1
Bos taurus	HM045018.1
Xenopus laevis	NC_001573.1

A total of 17 vertebrate mitochondrial DNA sequences were used in the interspecific analysis. These included *Bos Taurus*, *Cebus albifrons*, *Gorilla gorilla*, *Homo sapiens*, *Hylobates lar*, *Lemur catta*, *Macaca mulatta*, *Macaca sylvanus*, *Mus musculus*, *Nycticebus coucang*, *Pan paniscus*, *Pan troglodytes*, *Papio hamadryas*, *Pongo abelii*, *Pongo pygmaeus*, *Tarsius bancanus*, and *Xenopus laevis*.

of these participants ranged from 39 to 68 years, with a median age of 57 years.

Mutational Analysis of 22 Mitochondrial tRNA Genes

DNA fragments spanning 22 tRNA genes were PCR-amplified from genomic DNA of 80 affected Chinese subjects and 512 unaffected controls. Each fragment was purified and subsequently analyzed by DNA sequencing. Comparison of the resultant sequence with the Cambridge consensus sequence identified 16 (1 novel and 15 known) nucleotide changes in the 9 tRNA genes, as shown in Table 2. The novel variants were T7546C in $tRNA^{Asp}$, whereas the known variants were T4386C in $tRNA^{Gln}$, A5592G and C5601T in $tRNA^{Ala}$, G5821A in $tRNA^{Cys}$, A10005G and T10007C in $tRNA^{Gly}$, T10454C in $tRNA^{Arg}$, A12172G in $tRNA^{His}$, A14687G and A14693G in $tRNA^{Glu}$, T15889C, and G15927A, G15928A, G15930A, and A15951G in $tRNA^{Thr}$.[24] All the nucleotide changes were verified by sequence analysis of both strands and appeared to be homoplasmy. Among 80 subjects with coronary heart disease, 25 subjects carried 1 tRNA variant, whereas none of other 55 subjects harbored any mitochondrial tRNA variant.

Evaluation of Mitochondrial tRNA Variants

To identify putative deleterious mutation, these variants were further evaluated using the following 3 criteria: (1) present in <1% of the controls; (2) CI >75%, proposed by Ruiz-Pesini and Wallace[23]; and (3) potential structural and functional alterations. First, we used the secondary structure of tRNAs to localize each variant with either a stem or a loop and to analyze if the base changes within stems altered the classic Watson-Crick base pair. As shown in Figure 1, 9 variants were located at the loops, whereas 6 variants occurred in the stems of tRNAs. As shown in Table 4 and Figure 1, the A5592G variant in $tRNA^{Ala}$ created a putative C-G base-pairing, whereas variants G5821A in $tRNA^{Cys}$ and T15889C, G15927A, G15928A, and A15951G in $tRNA^{Thr}$ abolished putative base pairing(s). In addition, a phylogenetic analysis was performed by comparing the human tRNA nucleotide variants with those in 16 other vertebrates. As shown in Table 4, CI among the variants ranged from 12.5% ($tRNA^{Thr}$ G15930A variant) to 100% ($tRNA^{Ala}$ A5592G and $tRNA^{Glu}$ A14693G variants). In particular, the CI of 7 variants was >75%, the CI of 6 other variants was between 75% and 50%, and the CI of the remaining variants was <50%. These variants were then evaluated by examining the allelic frequency in 512 Han Chinese controls and 2704 control mtDNAs.[25] The T7546C variant was absent in both this Chinese control population and the 2704 mtDNAs. Five variants were absent in this cohort of Chinese controls and <1% in the 2704 mtDNAs, whereas the allelic frequency of 10 other variants was >1% in this control population and/or in the 2704 mtDNAs. Based on these criteria, the $tRNA^{Ala}$ A5592G and $tRNA^{Asp}$ T7546C mutations may have functional significance. In addition, our previous investigation showed that the $tRNA^{Thr}$ G15927G mutation led to a failure in tRNA metabolism.[26] Thus, these 3 tRNA variants are putative mutations associated with coronary heart disease. Furthermore, statistical analysis was carried out using Fisher's exact test to evaluate the difference in mitochondrial tRNA mutations between 80 subjects with CHD and 512 Chinese controls. The variants with $P<0.05$ were T15889C, G15927A, and G15930A, whereas the P value of 4 variants (A5592G, T7546C, A1005G, and T1007C) was 0.135. The higher P value of these 4 variants may be a result of the small sample size of subjects with CHD.

Clinical and Genetic Characterization of 7 Chinese Subjects Carrying 1 of the Putative Mutations Associated With Coronary Heart Disease

Ten probands and other members in these families carrying 1 of the putative mutations underwent physical examinations and laboratory assessments of cardiovascular disease risk

Table 3. Summary of Anthropometric, Clinical, and Biochemical Data of 80 Han Chinese Subjects With Coronary Heart Disease

Subjects	Sex	Age at Onset (year)	Age of Subject (y)	BMI	SBP/DBP (mm Hg)	Smokers	HT	LVH	DM	FBG (mmol/L)	TC (mmol/L)	TG (mmol/L)	HDL-C (mmol/L)	LDL-C (mmol/L)	Statins	Angina in Anamnesis	AMI in Anamnesis	Stroke in Anamnesis	Family Anamnesis for AMI	Family Anamnesis for HT	Family Anamnesis for T2DM
BJH1	M	76	78	21.22	120/80	Yes	No	No	No	5.31	6.03	1.1	1.46	4.35	Yes	Yes	No	Yes	No	Yes	No
BJH2	M	60	60	24.57	145/70	Yes	No	No	Yes	9.19	7.08	1.44	1.06	5.73	No	Yes	No	No	No	No	Yes
BJH3	M	45	46	25.16	160/100	Yes	Yes	Yes	No	4.08	6.93	1.7	1.66	4.93	No	No	No	No	No	Yes	No
BJH4	F	49	52	33.75	115/65	No	No	No	No	4.66	5.45	1.03	1.55	3.69	Yes	Yes	No	No	No	No	No
BJH5	F	52	57	25.81	135/100	No	Yes	No	No	4.44	6.65	1.21	0.99	5.42	No	Yes	No	No	No	No	No
BJH6	M	66	66	25.18	125/70	Yes	No	No	Yes	7.22	6.33	1.32	1.23	4.84	Yes	No	No	No	No	Yes	Yes
BJH7	F	70	73	23.92	160/100	No	Yes	Yes	No	5.06	6.41	1.08	1.45	4.74	Yes	Yes	Yes	No	No	Yes	No
BJH8	M	60	63	24.98	160/115	Yes	Yes	Yes	No	5.32	5.21	2.11	1.02	3.77	Yes	No	No	No	No	Yes	No
BJH9	M	76	77	24.57	170/100	Yes	Yes	Yes	Yes	7.56	5.66	1.65	0.85	4.48	Yes	Yes	No	No	No	No	Yes
BJH10	F	64	64	29.38	170/100	No	Yes	Yes	No	5.11	7.66	2.65	0.97	6.16	Yes	No	No	No	No	Yes	No
BJH11	F	65	68	23.42	150/90	No	Yes	No	Yes	5.52	6.33	2.66	1.66	4.14	Yes	Yes	No	No	No	No	No
BJH12	M	58	58	25.25	150/110	Yes	Yes	Yes	No	4.03	6.78	1.68	1.72	4.72	Yes	Yes	No	No	No	No	No
BJH13	M	56	60	23.14	150/120	Yes	Yes	Yes	No	4.12	5.45	1.25	1.54	3.66	Yes	No	No	No	No	No	No
BJH14	M	33	33	26.30	150/130	Yes	Yes	Yes	No	4.22	6.35	1.78	1.82	4.17	Yes	No	No	No	No	Yes	No
BJH15	M	50	48	24.69	170/90	Yes	Yes	Yes	No	4.35	7.12	2.31	1.02	5.64	Yes	Yes	Yes	No	Yes	No	No
BJH16	M	60	63	23.46	135/75	No	No	No	No	5.21	6.56	1.54	1.03	5.22	Yes	No	No	No	No	No	No
BJH17	M	58	59	25.01	160/100	No	Yes	Yes	Yes	7.54	5.68	1.68	1.87	3.47	Yes	Yes	Yes	No	No	No	No
BJH18	M	53	55	29.70	160/120	Yes	Yes	Yes	Yes	7.2	7.98	3.12	1.66	5.70	No	Yes	No	No	No	Yes	Yes
BJH19	M	52	48	26.99	130/80	Yes	No	No	Yes	15.5	6.85	1.89	1.11	5.36	Yes	No	No	No	No	No	No
BJH20	M	53	54	23.05	125/75	Yes	No	No	No	5.11	5.42	1.32	0.65	4.51	Yes	No	No	No	No	No	No
BJH21	M	58	60	21.97	145/100	Yes	Yes	No	Yes	24.2	6.85	1.56	0.87	5.67	Yes	No	No	No	No	No	No
BJH22	M	58	62	27.99	130/75	Yes	No	No	No	4.12	4.65	1.08	1.12	3.31	Yes	Yes	No	No	No	No	No
BJH23	M	75	75	24.21	125/75	No	No	No	No	4.32	5.12	1.32	1.66	3.20	Yes	Yes	No	No	No	Yes	No
BJH24	F	49	46	30.86	180/110	No	Yes	Yes	No	4.65	4.56	0.99	0.99	3.37	Yes	Yes	Yes	No	Yes	Yes	No
BJH25	M	60	62	22.86	140/80	Yes	No	No	No	5.23	5.12	1.02	1.54	3.38	Yes	No	No	No	No	No	No
BJH26	F	67	70	22.04	130/75	No	No	No	No	5.08	6.58	1.03	1.65	4.72	Yes	Yes	No	No	No	No	No
BJH27	M	49	55	26.09	180/110	Yes	Yes	Yes	No	5.12	5.11	1.65	1.32	3.46	Yes	Yes	No	No	No	Yes	Yes
BJH28	M	Died	Died	20.52	150/90	Yes	No	No	No	4.66	4.99	2.45	0.85	3.65	Yes	No	No	Yes	No	No	No
BJH29	M	70	70	24.16	180/90	Yes	Yes	Yes	No	4.87	4.58	2.12	1.66	2.50	Yes	Yes	No	No	No	Yes	No
BJH30	M	53	48	27.68	170/90	No	Yes	No	No	5.03	5.68	1.06	1.24	4.23	Yes	Yes	No	No	No	No	No
BJH31	F	69	70	23.28	180/90	No	Yes	Yes	No	4.66	4.21	3.12	0.99	2.60	Yes	Yes	No	No	No	Yes	Yes
BJH32	F	79	76	25.28	160/90	No	Yes	No	No	5.11	5.08	2.11	1.32	3.34	Yes	Yes	No	No	No	No	No
BJH33	M	50	55	22.53	170/90	Yes	Yes	Yes	Yes	8.9	4.26	1.04	1.34	2.71	Yes	Yes	No	No	No	Yes	No
BJH34	M	56	56	23.12	135/80	Yes	No	No	No	4.66	5.21	0.56	1.68	3.42	Yes	No	No	No	No	No	No
BJH35	M	59	60	29.54	180/110	Yes	Yes	Yes	Yes	7.31	5.12	0.85	1.45	3.50	Yes	Yes	No	No	No	Yes	Yes
BJH36	M	41	38	27.76	135/75	No	No	No	Yes	12.4	5.65	1.09	1.46	3.97	Yes	Yes	No	No	No	No	No
BJH37	M	58	58	26.57	160/110	No	Yes	Yes	No	5.18	4.87	1.45	1.35	3.23	Yes	Yes	Yes	No	No	Yes	Yes
BJH38	M	63	63	23.03	130/80	No	No	No	No	5.44	5.11	1.65	1.99	2.79	Yes	Yes	No	No	No	No	No
BJH39	M	63	66	27.76	125/75	Yes	No	No	Yes	5.12	6.03	1.45	0.56	5.18	Yes	Yes	No	No	No	No	Yes
BJH40	M	55	59	31.17	130/65	Yes	No	No	Yes	6.56	6.65	1.36	1.54	4.84	Yes	Yes	No	No	No	No	No
BJH41	M	Die	Die	25.71	180/120	Yes	Yes	Yes	No	4.69	5.65	2.08	1.27	3.96	Yes	Yes	No	Yes	No	Yes	No

Continued

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Table 3. Continued

Subjects	Sex	Age at Onset (year)	Age of Subject (y)	BMI	SBP/DBP (mm Hg)	Smokers	HT	LVH	DM	FBG (mmol/L)	TC (mmol/L)	TG (mmol/L)	HDL-C (mmol/L)	LDL-C (mmol/L)	Statins	Angina in Anamnesis	AMI in Anamnesis	Stroke in Anamnesis	Family Anamnesis for AMI	Family Anamnesis for HT	Family Anamnesis for T2DM
BJH42	M	79	79	24.16	160/90	No	Yes	No	No	5.44	5.12	1.03	1.37	3.54	Yes	No	No	Yes	No	No	No
BJH43	F	55	50	25.30	130/70	No	No	No	No	5.78	4.33	2.54	1.32	2.50	Yes	Yes	No	No	No	No	Yes
BJH44	F	67	69	23.34	160/80	No	Yes	No	No	4.99	5.12	1.66	1.46	3.33	Yes	Yes	No	No	No	Yes	No
BJH45	F	66	67	26.56	200/100	No	Yes	Yes	No	5.01	6.12	1.05	1.52	4.39	Yes	Yes	Yes	No	Yes	No	No
BJH46	M	61	63	22.38	130/75	No	No	No	No	5.65	4.86	1.79	1.51	2.99	Yes	Yes	No	No	No	No	No
BJH47	M	55	55	23.89	120/70	Yes	No	No	No	5.78	5.22	1.86	1.34	3.51	Yes	Yes	No	No	No	No	No
BJH48	M	50	46	25.71	150/90	No	Yes	No	No	4.98	5.36	1.03	0.65	4.50	Yes	NO	No	No	No	No	No
BJH49	F	59	57	27.03	160/100	No	Yes	No	No	4.68	4.69	1.05	1.23	3.25	Yes	Yes	No	No	No	Yes	No
BJH50	M	55	60	31.64	125/70	No	No	No	No	5.23	5.32	1.21	1.62	3.46	Yes	Yes	No	No	No	No	Yes
BJH51	M	56	58	22.02	130/70	Yes	No	No	No	5.42	6.03	1.31	1.25	4.52	Yes	Yes	No	No	No	No	No
BJH52	M	60	61	27.18	200/120	No	Yes	Yes	No	5.44	5.32	1.54	1.32	3.69	Yes	Yes	No	No	Yes	Yes	No
BJH53	F	73	73	26.11	220/160	No	Yes	Yes	Yes	7.65	4.96	0.85	1.54	3.25	Yes	No	No	No	No	Yes	No
BJH54	F	62	66	30.48	140/90	No	Yes	No	No	5.14	5.77	1.06	1.65	3.91	Yes	Yes	No	No	No	No	No
BJH55	M	49	53	25.06	230/180	Yes	Yes	Yes	No	5.41	4.99	0.99	1.24	3.55	Yes	Yes	No	No	No	Yes	No
BJH56	M	70	73	24.03	190/100	Yes	Yes	Yes	No	4.78	4.68	1.06	1.32	3.15	Yes	Yes	No	No	No	Yes	No
BJH57	M	69	69	26.33	170/100	Yes	Yes	Yes	Yes	14.1	5.32	1.54	1.62	3.39	Yes	No	No	No	No	No	No
BJH58	M	50	48	26.30	180/110	Yes	Yes	Yes	No	4.99	5.24	1.75	1.33	3.56	Yes	Yes	No	No	No	Yes	No
BJH59	F	53	57	18.03	135/70	No	No	No	No	4.85	4.12	1.63	1.24	2.55	Yes	Yes	No	No	No	No	No
BJH60	M	54	58	22.06	180/100	Yes	Yes	Yes	No	5.12	4.55	2.35	1.34	2.74	Yes	Yes	No	No	No	Yes	No
BJH61	M	62	65	25.47	145/95	Yes	No	No	No	5.21	5.21	1.55	1.24	3.66	Yes	No	No	No	No	No	No
BJH62	F	68	69	28.30	210/120	No	Yes	Yes	No	4.65	5.68	1.45	1.35	4.04	Yes	Yes	No	No	No	Yes	No
BJH63	M	76	78	19.81	200/120	No	Yes	Yes	Yes	7.3	4.32	1.68	1.65	2.33	Yes	Yes	No	No	No	Yes	Yes
BJH64	F	53	55	22.50	135/85	No	No	No	No	5.12	5.12	1.77	1.25	3.52	Yes	No	Yes	No	Yes	No	No
BJH65	M	64	65	26.96	160/100	Yes	Yes	No	No	5.32	5.24	1.69	1.38	3.52	Yes	Yes	No	No	No	Yes	No
BJH66	M	66	70	23.66	125/75	Yes	No	No	No	5.42	6.12	2.01	1.39	4.33	Yes	Yes	No	No	No	No	No
BJH67	M	47	49	26.89	160/90	Yes	Yes	No	No	5.21	5.47	1.54	1.41	3.75	Yes	Yes	No	No	No	Yes	No
BJH68	M	66	67	26.57	170/100	No	Yes	Yes	No	4.77	5.65	1.65	1.65	3.67	Yes	Yes	No	No	No	Yes	No
BJH69	F	51	51	23.34	180/100	No	Yes	Yes	No	4.85	4.89	1.87	1.34	3.18	Yes	Yes	No	No	No	Yes	Yes
BJH70	M	76	78	27.78	170/90	Yes	Yes	Yes	No	4.86	5.32	1.96	1.62	3.31	Yes	NO	No	No	No	Yes	No
BJH71	M	43	46	25.95	138/85	Yes	No	No	No	5.21	5.01	2.03	1.02	3.58	No	NO	No	No	No	No	No
BJH72	M	64	65	24.16	160/90	Yes	Yes	No	No	5.26	4.35	2.01	0.96	2.99	Yes	Yes	No	No	No	No	No
BJH73	M	53	55	26.03	220/120	Yes	Yes	Yes	No	4.98	5.21	1.54	1.35	3.55	Yes	Yes	No	No	No	Yes	No
BJH74	M	67	70	22.91	200/100	Yes	Yes	Yes	No	5.27	5.68	1.68	1.24	4.10	Yes	Yes	Yes	No	No	Yes	No
BJH75	F	53	53	27.55	135/85	No	No	No	No	5.68	5.45	1.58	1.35	3.78	Yes	Yes	No	No	No	No	Yes
BJH76	F	64	67	27.77	150/90	No	Yes	No	No	5.32	4.87	1.37	1.24	3.36	Yes	NO	No	No	Yes	No	No
BJH77	M	69	70	27.68	200/100	Yes	Yes	Yes	No	5.45	6.03	1.65	1.26	4.44	Yes	Yes	No	No	No	Yes	No
BJH78	F	72	72	22.58	180/100	No	Yes	Yes	No	4.89	5.31	1.54	1.25	3.75	Yes	Yes	No	No	No	Yes	Yes
BJH79	F	67	69	26.49	160/90	No	Yes	No	No	4.68	4.78	1.03	1.35	3.22	Yes	Yes	No	No	No	No	No
BJH80	M	54	55	27.44	130/70	No	No	No	Yes	7.8	5.12	0.65	1.25	3.74	No	Yes	No	No	No	No	Yes

AMI indicates acute myocardial infarction; BMI, body mass index; DBP, diastolic blood pressure; DM, diabetes mellitus; FBG, fasting blood glucose; HDL-C, high-density lipoprotein cholesterol; HT, hypertension; LDL-C, low-density lipoprotein cholesterol; LVH, left ventricular hypertrophy; SBP, systolic blood pressure; T2DM, diabetes mellitus type 2; TC, total cholesterol; TG, triglyceride.

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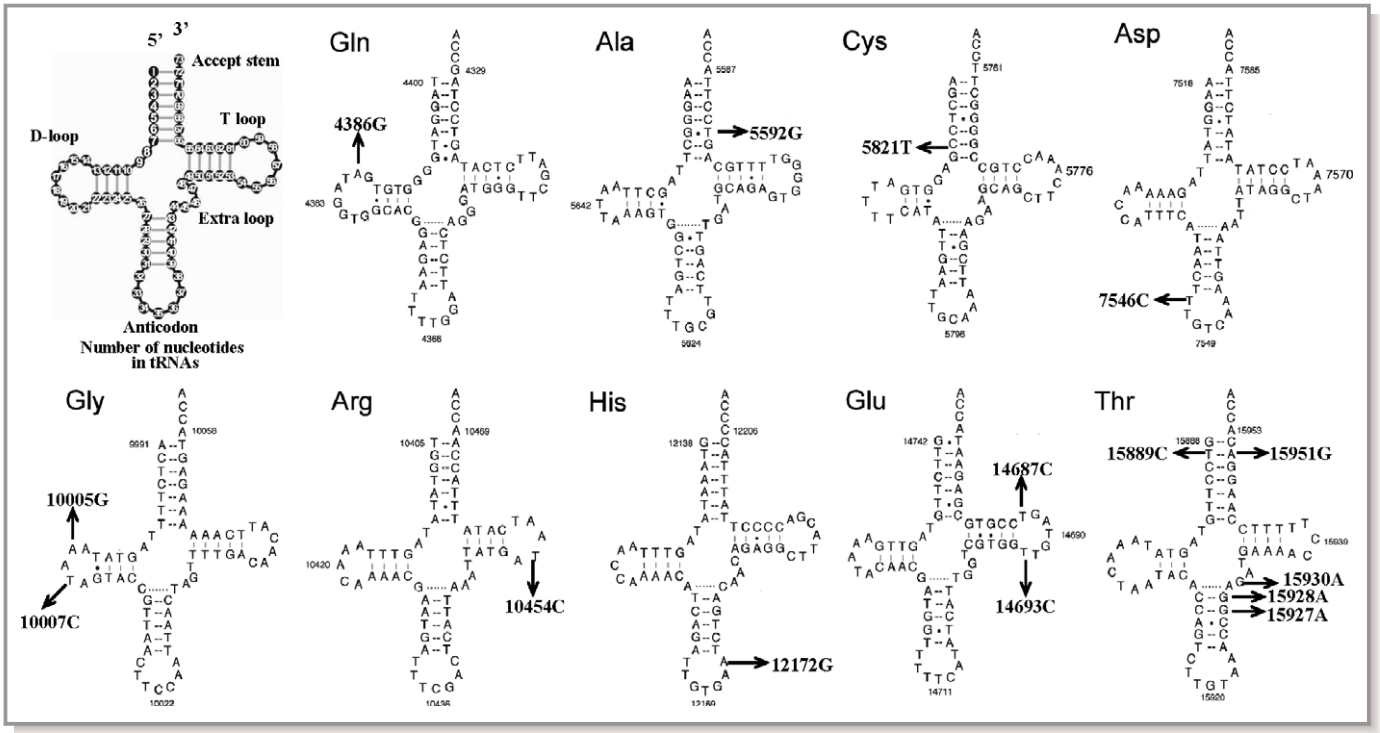

Figure 1. Mitochondrial tRNA variants in Chinese subjects with coronary heart disease. Cloverleaf structures of canonical tRNA and 9 mitochondrial tRNAs are shown. Circled numbers represent the nucleotide positions according to the conventional tRNA numbering system.[17] Tertiary interactions between nucleotides are indicated by dotted lines. Arrows indicate the position of the tRNA mutations.

Table 4. Variants in the Mitochondrial tRNA Genes in 80 Chinese Subjects With Coronary Heart Disease and 512 Controls

Genes	Position	Replacement	Conservation Index (%)*	WC Base Pairs†	Number of 80 Patients (%)	Number of 512 Controls (%)	χ^2 *P* Value	Number of 2704 mtDNAs‡ (%)	χ^2 *P* Value
$tRNA^{Gln}$	4386	T to C	75		1 (1.25)	4 (0.78)	0.517	51 (1.89)	1.000
$tRNA^{Ala}$	5592	A to G	100	C-G↑	1 (1.25)	0 (0)	0.135	3 (0.11)	0.110
	5601	C to T	50		2 (2.5)	15 (2.9)	0.709	37 (1.39)	0.309
$tRNA^{Cys}$	5821	G to A	62.5	C-G↓	1 (1.25)	12 (2.3)	1.000	14 (0.52)	0.355
$tRNA^{Asp}$	7546	T to C	100		1 (1.25)	0 (0)	0.135	0 (0)	0.029
$tRNA^{Gly}$	10005	A to G	87.5		1 (1.25)	0 (0)	0.135	3 (0.11)	0.110
	10007	T to C	43.8		1 (1.25)	0 (0)	0.135	4 (0.15)	0.136
$tRNA^{Arg}$	10454	T to C	50		1 (1.25)	4 (0.78)	0.517	11 (0.4)	0.269
$tRNA^{His}$	12172	A to G	93.8		1 (1.25)	5 (0.98)	0.583	31 (1.15)	0.609
$tRNA^{Glu}$	14687	A to G	93.8		1 (1.25)	1 (0.20)	0.252	22 (0.81)	0.490
	14693	A to G	100		2 (2.5)	7 (1.37)	0.349	10 (0.37)	0.045
$tRNA^{Thr}$	15889	T to C	18.8	U-A↓	2 (2.5)	1 (0.20)	0.049	3 (0.11)	0.008
	15927	G to A	68.8	G-C↓	4 (5)	7 (1.37)	0.048	44 (1.62)	0.047
	15928	G to A	68.8	G-C↓	1 (1.25)	2 (0.39)	0.354	132 (4.88)	0.181
	15930	G to A	12.5		4 (5)	5 (0.98)	0.023	37 (1.39)	0.029
	15951	A to G	56.25	A-U↓	1 (1.25)	2 (0.39)	0.354	22 (0.81)	0.490

*The conservation index (CI) was then defined as the percentage of the human nucleotide variants with 16 other vertebrates that had the wild-type nucleotide at that position.
†Classic Watson–Crick (WC) base pair: created (↑) or abolished (↓).
‡See http://www.genpat.uu.se/mtDB.

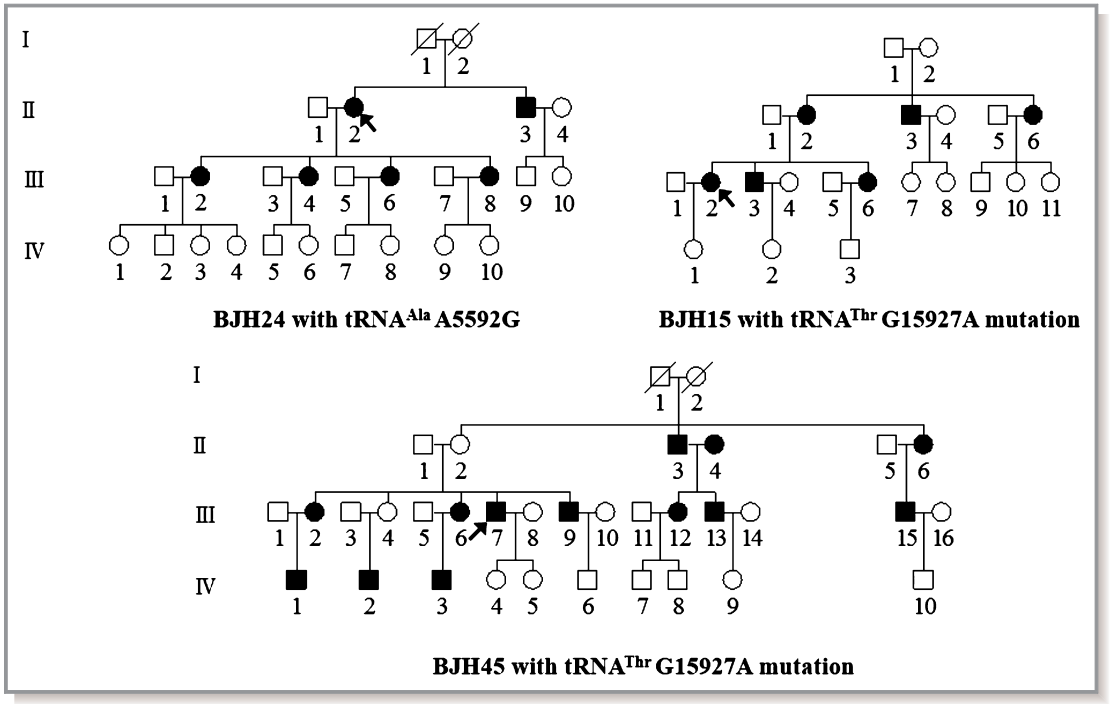

Figure 2. Three Han Chinese pedigrees with coronary heart disease. Affected individuals are indicated by filled symbols. An arrow denotes probands.

factors. Three probands, including subject BJH16 carrying the T7546C mutation and subjects BJH22 and BJH41 carrying the G15927A mutation, did not exhibit a family history of coronary heart disease. By contrast, 3 subjects had a family history of coronary heart disease. As shown in Figure 2, the pedigree of BJH24 carrying the A5592G mutation and the pedigrees of BJH15 and BJH45 harboring the G15927A mutation exhibited maternal transmission of coronary heart disease. In particular, 7 of 11 matrilineal relatives in the pedigree BJH24 and 6 of 17 matrilineal relatives in the pedigree BJH 45 suffered from coronary heart disease, whereas none of the affected fathers' offspring in these 2 families had clinical abnormalities. In the pedigree BJH15, 10 of 13 matrilineal relatives exhibited coronary heart disease, whereas all affected fathers with CHD, except subject II-3 who married affected subject II-4, never transmitted the trait to their offspring. These features are the maternal transmission of coronary heart disease in these 3 families.

Mutational Analysis of Mitochondrial Genomes

To assess the contribution that mtDNA variants or haplogroups make toward the phenotypic expression of these putative mtDNA mutations in these Chinese pedigrees, we performed PCR amplification of fragments spanning the entire mtDNA and subsequent DNA sequence analysis in 2 probands carrying the G15927A mutation and 1 proband carrying the A5592G mutation. The sequence results from these Chinese subjects were aligned with the updated consensus Cambridge sequence.[12] As shown in Table 5, these probands exhibited distinct sets of mtDNA polymorphisms. These included 27 variants in the D-loop region, 5 known variants in the 12S rRNA gene, 2 known variants in the 16S rRNA gene, the known $tRNA^{Gln}$ A5592G and $tRNA^{Thr}$ G15927A mutations, and the known NC7 9-bp deletion, as well as 29 (2 novel/27 known) silent variants and 11 known missense mutations in the polypeptide-encoding genes.[24] The mitochondrial genomes of subjects BJH15 and BJH45 belonged to the eastern Asian haplogroup B5b, whereas the mtDNA of subject BJH24 resided at haplogroup D4b.[27] These variants in RNAs and polypeptides were further evaluated by phylogenetic analysis of these variants and sequences from 16 other organisms including mouse,[28] bovine,[29] and *Xenopus laevis*.[30] Only the known CO1 G6969A (I356V) variant of subject BJH45 showed the high conservation in these species, proposed by Ruiz-Pesini and Wallace,[23] and had <1% frequency of 2706 mtDNAs. These data suggest that the CO1 G6962A(L356G) variant may have a role in the phenotypic manifestation of the G15927A mutation. By contrast, none of other variants showed both evolutionary conservation and <1% frequency of 2706 mtDNAs.

Discussion

In the present study, we performed a clinical, genetic, and molecular characterization of 80 Han Chinese subjects with

Table 5. mtDNA Variants in 3 Han Chinese Probands With Coronary Heart Disease

Gene	Position	Replacement	Conservation (H/B/M/X)*	CRS†	BJH15	BJH45	BJH24	Previously Reported
D-loop	73	A to G		A	G	G	G	Yes
	103	G to A		G	A	A		Yes
	152	T to C		T		C		Yes
	189	A to G		A		G		Yes
	199	T to C		T		C		Yes
	204	T to C		T		C		Yes
	263	A to G		A	G	G	G	Yes
	310	T to CT/CTC/CTCC		T	CTC	CT	TC	Yes
	315	C to CC		C		CC		Yes
	481	C to T		C	T			Yes
	489	T to G		T			G	Yes
	514	C to Del		C	Del C	Del C	Del C	Yes
	515	A to Del		A	Del A	Del A	Del A	Yes
	16111	C to T		C	T			Yes
	16140	T to C		T	C			Yes
	16183	A to C		A	C			Yes
	16189	T to C		T	C	C	C	Yes
	16193	C to CC		C	CC			Yes
	16223	C to T		C			T	Yes
	16234	C to T		C	T			Yes
	16243	T to C		T	C			Yes
	16344	C to T		C	T			Yes
	16362	T to C		T			C	Yes
	16463	A to G		A	G			Yes
	16519	T to C		T	C			Yes
	16569	T to C		T			C	Yes
12S rRNA	709	G toA	G/G/A/-	G	A	A		Yes
	750	A to G	A/A/A/-	A	G		G	Yes
	1382	A to C	A/A/A/G	A			C	Yes
	1438	A to G	A/A/A/G	A	G	G	G	Yes
	1598	G toA	G/A/T/T	G	A	A		Yes
16S rRNA	2626	T to C	T/T/A/G	T		C		Yes
	2706	A to G	A/G/A/A	A	G	G	G	Yes
ND1	4161	C to T		C	T			Yes
ND2	4769	A to G		A	G		G	Yes
	4883	C to T		C			T	Yes
	4895	A to G		A	G			Yes
	5178	C to A (Leu to Met)	L/T/T/T	C			A	Yes
$tRNA^{Ala}$	5592	A to G	A/A/A/A	A			G	Yes
CO1	6962	G to A (Leu to Gly)	L/L/L/L	G		A		Yes
	7028	C to T		C		T	T	Yes
CO2	8020	G to A		G			A	Yes

Continued

Table 5. Continued

Gene	Position	Replacement	Conservation (H/B/M/X)*	CRS†	BJH15	BJH45	BJH24	Previously Reported
NC7	8271-79	9-bp del		C	9-bp Del	9-bp Del		Yes
ATP8	8414	C to T (Leu to Phe)	L/F/M/W	C			T	Yes
ATP6	8584	G to A (Ala to Thr)	A/V/V/I	G	A	A		Yes
	8701	A to G (Thr to Ala)	T/S/L/Q	A			G	Yes
	8828	C to T		C		T		No
	8829	C to T		C	T			Yes
	8856	G to A		G		A		Yes
	8860	A to G (Thr to Ala)	T/A/A/T	A	G	G	G	Yes
	8964	C to T		C			T	Yes
CO3	9296	C to T		C			T	Yes
	9540	T to C		T			C	Yes
	9824	T to C		T			C	Yes
	9950	T to C		T	C			Yes
ND3	10398	A to G (Thr to Ala)	T/T/T/A	A	G	G	G	Yes
	10400	C to T		C			T	Yes
ND4	10873	T to C		T			C	Yes
	11101	A to G		A	G			Yes
	11719	G to A		G	A	A	A	Yes
ND5	12361	A to G		A	G	G		Yes
	12705	C to T		C			T	Yes
	13959	C to T		C			T	No
ND6	14221	T to C		T	C			Yes
	14668	C to T		C			T	Yes
Cytb	14766	C to T (Thr to Ile)	T/S/T/S	C	T	T	T	Yes
	14783	T to C		T			C	Yes
	15043	G to A		G			A	Yes
	15223	C to T		C	T	T		Yes
	15301	G to A		G			A	Yes
	15326	A to G (Thr to Ala)	T/M/I/I	A	G	G	G	Yes
	15508	C to T		C	T	T		Yes
	15662	A to G (Ile to Val)	I/L/F/L	A	G	G		Yes
	15850	T to C		T	C			Yes
	15851	A to G (Ile to Val)	I/A/S/M	A	G	G		Yes
$tRNA^{Thr}$	15927	G to A	G/G/G/G	G	A	A		Yes

CRS indicates Cambridge reference sequence.[5]

*Conservation of amino acid for polypepides or nucleotide for RNAs in human (H), bovine (B), mouse (M), and *Xenopus laevis* (X).

†See the online mitochondrial genome database http://www.mitomap.org and http://www.genpat.uu.se/mtDB/

coronary heart disease. Mutational analysis of mitochondrial tRNA genes identified 16 variants. These variants were further evaluated using the following criteria: (1) present in <1% of the controls; (2) evolutional conservation; (3) potential structural and functional alterations; (4) maternal transmission of coronary heart disease in matrilineal relatives carrying 1 of tRNA mutations. Of these variants, only $tRNA^{Gln}$ A5592G and $tRNA^{Thr}$ G15927A mutations were fitted with these criteria, suggesting that these mutations may be associated with coronary heart disease.

The first evidence was that the pedigrees of BJH24 carrying the A5592G mutation, BJH15, and BJH45 harboring the

G15927A mutation exhibited maternal transmission of coronary heart disease. In particular, 7 of 11 matrilineal relatives in the pedigree BJH24, 6 of 17 matrilineal relatives in the pedigree BJH45, and 10 of 13 matrilineal relatives of pedigree BJH15 suffered from coronary heart disease, whereas none of affected fathers' offspring in these families had clinical abnormalities. The maternal transmissions of coronary heart disease in these 3 families strongly suggest that mutations in mitochondrial DNA are the molecular basis of this disorder in these families. Further sequence analysis of their mitochondrial genomes confirmed the presence of the homplasmic A5592G or G15927A mutations in all matrilineal relatives but not other members of these families. The absence of functionally significant mutations in mtDNAs of probands BJH15-III-2 and BJH24-II-2 indicates that mitochondrial backgrounds may not play an important role in the pathogenesis of coronary heart disease. On the other hand, the CO1 G6962A (L356G) variant may have a role in the phenotypic manifestation of the G15927A mutation in the pedigree of BJH45.

The T5592C mutation is localized at a highly conserved uridine (68U) on the acceptor stem of $tRNA^{Ala}$, where the position is important for the stability and identity of tRNA.[17,31] The U-to-C transition at this position by the T5592C mutation is expected to create a highly conservative base pairing (5G-68C) on the acceptor stem of this tRNA, alter the secondary structure of this tRNA, as in the case of the deafness-associated $tRNA^{His}$ T12201C mutation.[32] Our previous investigation showed that the T12201C mutation destablized a highly conservative base pairing (5A-68U) on the acceptor stem of this tRNA, leading to a failure in tRNA metabolism.[32] In particular, ≈70% decrease in $tRNA^{His}$ steady-state level was observed in mutant cells carrying the T12201C mutation, compared with those of controls. These observations further supported the functional significance of the T5592C mutation in the pedigree BJH45 with coronary heart disease.

The homoplasmy G15927A mutation is at a highly conserved nucleotide (G42) in the anticodon stem of $tRNA^{Thr}$, where the position is important for the stability and identity of tRNA.[31] The anticipated destabilization of base pairing (28C-42G) by the G15927A mutation affects secondary structure and function of this tRNA, as in the cases of $tRNA^{Ile}$ A4300G and $tRNA^{Leu(UUR)}$ T3273C mutations.[33,34] The G15927A mutation changed the conformation of $tRNA^{Thr}$, as suggested by slower electrophoretic mobility of mutated tRNA with respect to the wild-type molecule. However, the aminoacylation level of the $tRNA^{Thr}$ was not impaired, but the steady-state level of tRNA was reduced 44% in lymphoblastoid cell lines derived from Chinese control subjects carrying the G15927A mutation.[26] The alteration in tRNA metabolism by the G15927A mutation impaired mitochondrial translation and respiration, increasing the production of reactive oxygen species.[35]

The homoplasmic nature of the mtDNA A5592G and G15927A mutations hints at the mild nature of the mutations. These suggest that these mtDNA mutations are by themselves insufficient to produce a clinical phenotype[19,20,36] but the inherited risk factors are necessary for the development of coronary heart disease. The nuclear modifier genes and environmental and epigenetic factors, as well as personal lifestyles, may also contribute to the development of coronary heart disease in these subjects carrying the mtDNA mutation.[37–40] In particular, the tissue specificity of the $tRNA^{Ala}$ A5592G or $tRNA^{Thr}$ G15927A mutation in these Chinese families is likely attributed to tissue-specific tRNA metabolism or the involvement of nuclear modifier genes.

In summary, our data provide evidence that mitochondrial genetic defects may lead to coronary heart disease. The mitochondrial $tRNA^{Ala}$ A5592G and $tRNA^{Thr}$ G15927A mutations altered the structure and function of their tRNAs, thereby causing mitochondrial dysfunctions and long-standing increase of reactive oxygen species in cardiovascular cells. These 2 mutations may be the inherited risk factors for coronary heart disease. Thus, our findings may provide new insights into the understanding of the pathophysiology and valuable information for the management and treatment of coronary heart disease.

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Disclosures

None.

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