Maternally inherited coronary heart disease is associated with a novel mitochondrial tRNA mutation

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Abstract

Background: Coronary heart disease (CHD) is the most common cause of mortality globally, yet mitochondrial genetic mutations associated with CHD development remain incompletely understood. Objective: To investigate the mitochondrial tRNA mutation associated with CHD. Methods: We are conducting ongoing systematic screening efforts assessing mtDNA mutations among Chinese CHD patients. And we followed up on its biological significance in cybrid cell lines bearing this mutation, measuring the effects of this 15910C>T mutation on tRNAThr levels, enzymatic activity of electron transport chain complexes, membrane permeability, and the mitochondria-mediated generation of both ROS and ATP. Results: In the present report, we characterize mitochondrial genetic mutations in a three-generation Chinese family exhibiting signs of maternally inherited CHD. Of the 24 different family members in this pedigree we assessed, CHD was detected in 6, with variable severity and age of first appearance. When we sequenced the mitochondrial genomes of these individuals, we found a tRNAThr 15910C>T mutation of the Eastern Asian haplogroup M7b’c. This mutation is predicted to destabilize the strongly conserved (24C-10G) base-pairing, thereby disrupting tRNAThr functionality. When we performed Northern blotting, we detected we observed a 37.5% reduction in tRNAThr levels at baseline in cells bearing the 15910C>T mutation. When we conducted western blot analysis, we detected a ~24.96% decrease in mitochondrial translation rates in these same cells. Conclusions: Together these findings suggest a possible link between this 15910C>T tRNAThr mutation and CHD, potentially offering new avenues for future disease intervention.

Background

Different Cardiovascular diseases remain the most prominent cause of death globally, with
coronary heart disease (CHD) remaining a highly complex and heterogeneous disease the
onset of which is typically influenced by a range of environmental and genetic factors,
although in some cases single gene mutations can drive disease\textsuperscript{1, 2}. While many studies to
date have sought to identify nuclear genomic factors linked with CHD incidence, relatively
few studies have specifically investigated CHD risk arising as a consequence of
mitochondrial mutations\textsuperscript{3-5}. Such investigations are important, as abnormal mitochondrial
functionality has been found to be a potential driver of hypertension and other
cardiovascular diseases\textsuperscript{6, 7}. Multiple previous studies have identified linked between
specific mitochondrial DNA (mtDNA) mutations and hypertension, including the tRNA\textsuperscript{Ile}
4295A>G mutation, as well as the tRNA\textsuperscript{Ile} 4263A>G, tRNA\textsuperscript{Met}/tRNA\textsuperscript{Gln} 4401A>G, and
tRNA\textsuperscript{Met} 4435A>G mutations which were specifically linked to hypertension in Chinese
individuals\textsuperscript{8-11}.

To explore additional mutations linked with CHD pathogenesis, we are conducting ongoing
systematic screening efforts assessing mtDNA mutations among Chinese CHD patients. As
a part of this effort, we have identified one three-generation family presenting with
evidence of CHD transmitted matrilineal, with 6/24 analyzed adults in this family
exhibiting CHD of varying severity. When we analyzed their mtDNA, we detected the
presence of a tRNA\textsuperscript{Thr} 15910C>T mutation of the M7b’c haplogroup. This mutation
occurred in the stem region of this tRNA (conventional position 25) a site that is highly
conserved and the mutation of which is predicted to result in structural and functional
changes that have the potential to disrupt normal mitochondrial functionality. After
identifying this mutation we followed up on its biological significance in cybrid cell lines
bearing this mutation, measuring the effects of this15910C>T mutation on tRNA\textsuperscript{Thr} levels,
enzymatic activity of electron transport chain complexes, membrane permeability, and the mitochondria-mediated generation of both reactive oxygen species (ROS) and adenosine triphosphate (ATP).

Methods

Subjects

For this study, a three-generation Han Chinese family (Q5) affected by CHD were analyzed at affiliated hospital of Qingdao university. In addition, 113 unrelated controls were also obtained from among volunteers in the same area. All participants provided informed consent, and underwent both clinical evaluation and blood sample collection. The Qingdao University ethics committee oversaw and approved this study.

Assessment of risk factors

Relevant risk factors in the present study included hypertension, hyperlipidemia, diabetes, a history of smoking, or a family history of CHD. Patient blood pressure was measured according to standard methods using the average of three readings. Hypertension was designated as a systolic blood pressure ≥140 mmHg and/or a diastolic blood pressure ≥90 mmHg as per JNC VI criteria. Diabetes was diagnoses in patients based on the presence of either a need for antidiabetic medications, or fasting blood glucose >126 g/dL, as in previous reports. Any individuals who reported having used cigarettes within the past 12 months were designated smokers.

Mitochondrial mutational assessment

The Puregene DNA Isolation Kits (Biomega) was used to isolate total genomic DNA from study participants, after which mitochondrial genomic DNA was assessed via Southern blotting as in previous research. A total of 24 overlapping PCR fragments were generated and amplified in order to provide full coverage of the mitochondrial genome, using appropriate pairs of light/heavy strand primers used in previous studies. An ABI 3700
automated DNA sequencer was then employed to sequence each of these fragments following purification with a Big Dye Terminator Cycle sequencing reaction kit. The consensus Cambridge sequence (GenBank accession number: NC_012920) was then used for alignment of these sequenced fragments. Detection of the 15910C>T mutation in family members, 80 unrelated CHD patients, and other controls was performed as in previous studies.

**Cell culture**

The Epstein-Barr virus was used to generate immortalized patient cell lines from the proband patient (III-3) bearing the 15910C>T mutation, as well as from a control individual (C2). These cells were cultured in RPMI 1640 containing 10% FBS. Cybrid cells were generated by adapting previous protocols. Briefly, bromodeoxyuridine (BrdU)-resistant 143B.TK⁻ cells were cultured in DMEM containing 5% FBS, and the ρ0206 cell line lacking mtDNA derived from these same cells was also grown under these conditions in the presence of 50 μg uridine/ml. The patient and control cell lines were then enucleated and fused with the ρ0 206 cells. The resultant cybrid cell lines were then selected in uridine-free DMEM supplemented with BrdU, allowing for donor-derived cybrid lines that could then be assessed for the m.15910C>T mutation, amounts of mtDNA, and other cellular genetic features. The resultant cybrid lines were maintained in DMEM containing 5% FBS.

**Assessment of mitochondrial tRNA levels**

TRIzol was used to isolate total mitochondrial RNA from knockdown or control cell lines, as in previous studies¹², and tRNA modifications were then assessed based on changes in the electrophoretic mobility of these tRNAs through a polyacrylamide gel containing 0.05 mg/ml APM. In total, we separated 10 mg of total RNA electrophoretically, followed by
transfer onto a positively charged membrane which was then combined with appropriate DIG oligodeoxynucleoside probes based on previously described approaches, using tRNA<sup>Thr</sup>, tRNA<sup>His</sup>, tRNA<sup>Ala</sup>, and tRNA<sup>Glu</sup> utilized in previous studies.

**Western blotting**

Western blotting was used to assess protein levels in cells, via first electrophoretically separating 20 ug of protein from each sample via SDS-PAGE. These samples were then transferred onto PVDF membranes, which were then probed with primary antibodies against ND4, ND1, ND6, CYTB, ATP6, CO2, and VDAC. Secondary Affini Pure goat anti-mouse IgG and goat anti-rabbit IgG conjugated to peroxidase enzymes were then used to probe these blots, followed by use of an ECL system for chemiluminescent detection. Densitometric band quantification was then performed as in previous studies.

**Measurements of enzymatic activity**

The complex I, II, III, and IV activities were assessed as in previous studies<sup>12</sup>.

**Measuring ATP levels**

In order to assess ATP generation in cells, the Cell Titer-Glo Luminescent Cell Viability Assay kit (Promega) was used based on provided protocols<sup>12</sup>.

**Mitochondrial membrane potential measurements**

The JC-10 Assay Kit (Abcam) was used to measure mitochondrial membrane potential based on provided protocols.

**Assessment of ROS levels**

The MitoSOX Red Mitochondrial Superoxide Indicator (Invitrogen, M36008) was used to assess ROS production in live cells based on provided protocols.

**Statistical Analysis**

Unpaired, two-tailed Student’s t-tests were used to compare all values in this study. SPSS
Results

Clinical presentation

The proband (Q5-III-3) was first diagnosed with CHD upon presenting at the Cardiology Clinic of affiliated hospital of Qingdao university at age 40, after which he underwent a full medical evaluation. The patient was diagnosed with hypertension (159/99 mmHg), significant ischemia (65% narrowing was evident upon coronary angiography), and high cholesterol (LDL-C = 159 mg/dL, TC = 232 mg/dL). The patient was not affected by any other comorbid conditions such as diabetes or neurological disease. When family members of this patient were evaluated for these same conditions, 5 were diagnosed with all three of these conditions (Figure 1a and Table 1). In each case, any fathers with CHD had not transmitted it to their children, whereas mothers did transmit it, suggesting matrilineal inheritance consistent with mitochondrial involvement in this inherited CHD risk.

Analysis of mitochondrial mutations

To explore potential mitochondrial mutations linked with inherited CHD risk, we sequenced the mitochondrial genome of this proband patient Q5-III-3. A total of 45 mutations were evident in their mitochondrial genome upon comparison with the Cambridge consensus sequence, and the mitochondrial haplogroup for this patient was identified to be M7b’c (Figure 2). As shown in Table 2, of these 45 variants, 19 were known silent variants, 14 were known D-loop variants, 8 were known missense mutations affecting protein-coding genes, 2 were known 12S rRNA variants, 1 was a known 16s rRNA variant, and one was a novel homoplasmy 15910C>T mutation in the tRNAThr gene (Figure 1B). The detected missense mutations were as follows: 5460G>A (Ala331Thr) in the ND2 gene, 7853G>A
(Val90Ile) in the CO2 gene, the 8701A>G (Thr59Ala) in the ATP6 gene, the 10398A>G (Thr114Ala) in the ND3 gene, 12811T>C (Tyr159His) in the ND5 gene, the 14766C>T (Thr>Ile), 14978A>G (Ile78Val), and m.15326A>G (Thr>Ala) in the CYTB gene. We compared the variance at these mutated RNA residues phylogenetically across 16 different primate species, revealing this tRNA<sup>Thr</sup> 15910C>T mutation to have a 100% conservation index across species, making it more likely to have functional significance when mutated as in this patient. This mutation was also not detected when 136 Chinese control subjects were analyzed.

**Mutation leads to decreased mitochondrial tRNA<sup>Thr</sup> levels**

We next assessed how this 15910C>T mutation altered the metabolism of tRNA<sup>Thr</sup>, subjecting cybrid cell lines bearing this mitochondrial mutation to Northern blotting using probes specific to this and 3 other tRNAs. We found that tRNA<sup>Thr</sup> levels in these mutant cybrid lines were significantly reduced relative to control wild type cells (Figure 2), with baseline tRNA<sup>Thr</sup> levels in these mutant cells being 65.25% of those in control cells, with 5S RNA used for normalization purposes. In contrast, baseline tRNA<sup>His</sup>, tRNA<sup>Ala</sup>, and tRNA<sup>Glu</sup> levels in these mutant cell lines were unchanged relative to control cells (102.13%, 98.89%, and 107.91%, respectively).

**Mutation leads to reduced mitochondrial protein levels**

We next performed Western blotting to assess levels of the mtDNA-encoded components of the respiratory complex in cells bearing the 15910C>T mutation or controls. As shown in Figure 3, we found that mutant cells expressed mitochondrial protein levels that were 19.31% to 31.55% of those in control cells (average =24.96%; P<0.05). These mutated cells also showed clear reductions (18.71%, 26.56%, 37.52%, 33.00% and 39.48%) in 5 polypeptides (ND4, ND1, ND6, CYTB and ATP6), while CO2 levels were not significantly
reduced (0.15%) relative to control cells.

**Mutation led to decreased complex I and III activity**

We further assessed the consequences of this m.15910C>T mutation on oxidative phosphorylation using isolated mitochondrial from our mutant and control cybrid cell lines. We found that complex I and III activity in the 15910C>T mutant mitochondria was 66.72%, and 75.48% that of the activity observed in control cells, whereas no changes in complex II/IV activity were observed (Figure 4A).

**Mutation leads to reduced mitochondrial ATP generation**

We further assessed the generation of ATP by wild type and mutant cells in an effort to better gauge how this mutation influenced oxidative phosphorylation. To test this, either glycolysis or oxidative phosphorylation were selectively induced in cells via culture with glucose, glucose + oligomycin, or 2deoxy-D-glucose + pyruvate. When cells could only engage in oxidative phosphorylation, mutant cells bearing the 15910C>T mutation exhibited ATP production that was 65.68 - 67.98% (average: 67.37%) of that in control cells (Figure 4B).

**Mutation leads to increased ROS production**

We next assessed ROS production in our mutant cybrid cell lines via flow cytometry, comparing baseline staining intensity for each cell line to that upon oxidative stress in order to obtain a ratio corresponding to ROS generation. We observed somewhat increased ROS generation for our mutant cybrid lines bearing the 15910C>T mutation, with ROS production 118.45 - 123.98%, (average: 121.04%) that of control cells (Figure 4C).

**Discussion**

In this study, we offer evidence of a novel mitochondrial mutation which is linked to an elevated risk of CHD. This mutation was detected among adult matrilineally-related individuals in a three-generation Chinese family, with affected individuals presenting with
CHD, hypertension and hyperlipidemia. This CHD risk was matrilineally inherited, and a mutational analysis revealed the presence of a 15910C>T mutation at the C25 position in the tRNA\textsuperscript{Thr} sequence - a residue which is normally highly conserved and which is predicted to be important for tRNA stability (Figure 1C). This mutations is predicted to destabilize the base-pairing at this site (25C-10G), potentially altering the secondary structure of this tRNA, as has previously been reported for the tRNA\textsuperscript{Ile} 4300A>G and tRNA\textsuperscript{Leu(UUR)} 3273T>C mutations\textsuperscript{13,14}.

When cybrid cells bearing this mutation were generated, their baseline tRNA\textsuperscript{Thr} levels were reduced by 37.5% relative to healthy control cells, suggesting that there may be a resultant destabilization of this mutated tRNA\textsuperscript{Thr} resulting in its more rapid degradation, as previously described for the 3243A>G mutation of tRNA\textsuperscript{Leu(UUR)}\textsuperscript{15-17}. As the mitochondrial dysfunction stemming from the 15910C>T mutation was relatively mild, this suggests that this mutation alone is unlikely to cause CHD. We observed a ~24.96% reduction in mitochondrial protein levels in cells bearing this mutation, and these cells als exhibited altered complex I/III activity which may coincide with increased electron leakage and elevated ROS production. Indeed, consistent with this we found that ROS production was elevated in cybrids expressing this 15910C>T mutation. Such ROS production can lead to significant damage to cellular macromolecules including DNA and proteins, potentially leading to cellular dysfunction or apoptotic cell death which, if it were to occur in cardiac muscle cells, could contribute to the observed CHD phenotype, potentially explaining how these mutations contribute to the observed matrilineal CHD, as mitochondrial tRNA associated with hypertension detailed previously\textsuperscript{18-21}.

As the 15910C>T mutation was homoplasmic in nature in the study subjects, this suggests that the mutation is relatively mild, consistent with the limited changes in mitochondrial
functionality observed herein. Even so, our study suggests that this gene mutation is linked to an elevated risk of CHD development, with the ultimate odds of CHD development likely depending on a combination of environmental, lifestyle, and nuclear genetic factors in concert with the observed mitochondrial dysfunction. Indeed, other mutations in nuclear genes may also contribute to mitochondrial dysfunction in patients bearing the 15910C>T mutation, potentially resulting in the observed CHD phenotype.

In conclusion, our results suggest that the mitochondrial tRNA\textsuperscript{Thr} 15910C>T mutation is linked to CHD incidence. This mutation leads to altered metabolism of this particular tRNA, thus resulting in abnormal mitochondrial functionality and enhanced ROS production. Other nuclear genetic mutations may also act in concert with the 15910C>T mutation to amplify consequent mitochondrial dysfunction in affected patients, and extended ROS production in cardiovascular cells may be linked to CHD onset. Our results thus suggest a potential new mechanism liked to the underlying pathology of CHD, indicating future avenues for therapeutic research.

Abbreviations

ATP: adenosine triphosphate

CHD: Coronary heart disease

ROS: Reactive oxygen species

Declarations

**Ethics approval and consent to participate**

All subjects were willing to participate in the study and the written informed consent for clinical evaluations and genetic analysis were obtained from each participant. In addition, the protocol of the study was approved by the medical ethics committee of the Qingdao University.
Consent for publication

Written informed consent to publish this information was obtained from study participants. All the data are available for the consultation.

Availability of data and material

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Funding

Not applicable.

Authors' contributions

HL and ML designed the experiments. XZ and JH collected the blood samples and extracted DNA from the blood samples. ZZ and YC analyzed the raw data. ZZ and HL wrote the manuscript. HL participate in revising the manuscript. All authors read and approved the final manuscript.

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Reference

1. Wang T, Chen L, Yang T, Huang P, Wang L, Zhao L, Zhang S, Ye Z, Chen L, Zheng Z, Qin J. Congenital Heart Disease and Risk of Cardiovascular Disease: A Meta-Analysis of Cohort Studies. J Am Heart Assoc. 2019;8(10):e012030.

2. Séguro F, Rabès JP, Taraszkiewicz D, Ruidavets JB, Bongard V, Ferrières J. Genetic diagnosis of familial hypercholesterolemia is associated with a premature and high coronary heart disease risk. Clin Cardiol. 2018;41(3):385-391.

3. Jia Z, Zhang Y, Li Q, Ye Z, Liu Y, Fu C, Cang X, Wang M, Guan MX. A coronary artery disease-associated tRNAThr mutation altered mitochondrial function, apoptosis and
angiogenesis. Nucleic Acids Res. 2019;47(4):2056-2074.

4. Humphries SE, Drenos F, Ken-Dror G, Talmud PJ. Coronary heart disease risk prediction in the era of genome-wide association studies: current status and what the future holds. Circulation. 2010;121(20):2235-48

5. Hegele RA. Genetic susceptibility to heart disease in Canada: lessons from patients with familial hypercholesterolemia. Genome. 2006;49(11):1343-50.

6. Corral-Debrinski M, Shoffner JM, Lott MT, Wallace DC. Association of mitochondrial DNA damage with aging and coronary atherosclerotic heart disease. Mutat Res. 1992;275(3-6):169-80.

7. Yang W, Ng FL, Chan K, Pu X, Poston RN, Ren M, An W, Zhang R, Wu J, Yan S, Situ H, He X, Chen Y, Tan X, Xiao Q, Tucker AT, Caulfield MJ, Ye S. Coronary-Heart-Disease-Associated Genetic Variant at the COL4A1/COL4A2 Locus Affects COL4A1/COL4A2 Expression, Vascular Cell Survival, Atherosclerotic Plaque Stability and Risk of Myocardial Infarction. PLoS Genet. 2016;12(7):e1006127.

8. Li Z, Liu Y, Yang L, Wang S, Guan MX. Maternally inherited hypertension is associated with the mitochondrial tRNA(Ile) A4295G mutation in a Chinese family. Biochem Biophys Res Commun. 2008;367(4):906-11.

9. Liu Y, Li R, Li Z, Wang XJ, Yang L, Wang S, Guan MX. Mitochondrial transfer RNAMet 4435A>G mutation is associated with maternally inherited hypertension in a Chinese pedigree. Hypertension. 2009;53(6):1083-90.

10. Li R, Liu Y, Li Z, Yang L, Wang S, Guan MX. Failures in mitochondrial tRNAMet and tRNAGln metabolism caused by the novel 4401A>G mutation are involved in essential hypertension in a Han Chinese Family. Hypertension. 2009;54(2):329-37.

11. Wang S, Li R, Fettermann A, Li Z, Qian Y, Liu Y, Wang X, Zhou A, Mo JQ, Yang L, Jiang P, Taschner A, Rossmanith W, Guan MX. 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. 2011;108(7):862-70.

12. Zhou M, Wang M, Xue L, Lin Z, He Q, Shi W, Chen Y, Jin X, Li H, Jiang P, Guan MX. A hypertension-associated mitochondrial DNA mutation alters the tertiary interaction and function of tRNALeu(UUR). J Biol Chem. 2017;292(34):13934-13946.

13. Taylor RW, Giordano C, Davidson MM, d'Amati G, Bain H, Hayes CM, Leonard H, Barron MJ, Casali C, Santorelli FM, Hirano M, Lightowlers RN, DiMauro S, Turnbull DM. A homoplasmic mitochondrial transfer ribonucleic acid mutation as a cause of maternally inherited hypertrophic cardiomyopathy. J Am Coll Cardiol. 2003;41(10):1786-96.

14. Campos Y1, Gámez J, García A, Andreu AL, Rubio JC, Martín MA, del Hoyo P, Navarro C, Cervera C, Garesse R, Arenas J. A new mtDNA mutation in the tRNA(Leu(UUR)) gene associated with ocular myopathy. Neuromuscul Disord. 2001;11(5):477-80.

15. Hung PC, Wang HS, Chung HT, Hwang MS, Ro LS. Pulmonary hypertension in a child with mitochondrial A3243G point mutation. Brain Dev. 2012;34(10):866-8.

16. Liu CH, Chang CH, Kuo HC, Ro LS, Liou CW, Wei YH, Huang CC. Prognosis of symptomatic patients with the A3243G mutation of mitochondrial DNA. J Formos Med Assoc. 2012;111(9):489-94

17. Lu J, Wang D, Li R, Li W, Ji J, Zhao J, Ye W, Yang L, Qian Y, Zhu Y, Guan MX. Maternally transmitted diabetes mellitus associated with the mitochondrial tRNA(Leu(UUR)) A3243G mutation in a four-generation Han Chinese family. Biochem Biophys Res Commun. 2006;348(1):115-9.

18. Xue L, Wang M, Li H, Wang H, Jiang F, Hou L, Geng J, Lin Z, Peng Y, Zhou H, Yu H, Jiang P, Mo JQ, Guan MX. Mitochondrial tRNA mutations in 2070 Chinese Han subjects with hypertension. Mitochondrion. 2016;30:208-21.
19. Jiang P, Wang M, Xue L, Xiao Y, Yu J, Wang H, Yao J, Liu H, Peng Y, Liu H, Li H, Chen Y, Guan MX. A Hypertension-Associated tRNAAla Mutation Alters tRNA Metabolism and Mitochondrial Function. Mol Cell Biol. 2016;36(14):1920-30.

20. Lin L, Cui P, Qiu Z, Wang M, Yu Y, Wang J, Sun Q, Zhao H. The mitochondrial tRNAAla 5587T>C and tRNALeu(CUN) 12280A>G mutations may be associated with hypertension in a Chinese family. Exp Ther Med. 2019;17(3):1855-1862.

21. Liu Y, Li Y, Zhu C, Tian L, Guan M, Chen Y. Mitochondrial biogenesis dysfunction and metabolic dysfunction from a novel mitochondrial tRNAMet 4467 C>A mutation in a Han Chinese family with maternally inherited hypertension. Sci Rep. 2017;7(1):3034.

Tables

Table 1 Summary of clinical data for some members in a Chinese pedigree

| Subjects | Gender | Age of onset (years) | Systolic pressure/Diastolic pressure (mmHg) | ECG | Extent of CAD narrow (%) | total cholesterol (mg/dl) | LDL (mg/dl) |
|----------|--------|----------------------|--------------------------------------------|-----|--------------------------|--------------------------|-------------|
| II-6     | F      | 62                   | 150/95                                      | ischemia | 55                       | 190                      | 140         |
| II-10    | F      | 60                   | 140/90                                      | ischemia | 50                       | 220                      | 145         |
| II-11    | M      | 55                   | 138/85                                      | ischemia | 50                       | 210                      | 150         |
| III-3    | M      | 40                   | 159/99                                      | ischemia | 65                       | 232                      | 159         |
| III-6    | M      | 35                   | 145/90                                      | ischemia | 50                       | 187                      | 140         |

Table 2 mtDNA variants in a Chinese family with CHD

| Gene | Position | Replacement | AA change | Conservation | Previously reported |
|------|----------|-------------|-----------|--------------|---------------------|
| D-loop | 73 | A-G | | Yes | |
|        | 143 | G-A | | Yes | |
|        | 150 | C-T | | Yes | |
|        | 199 | T-C | | Yes | |
|        | 204 | T-C | | Yes | |
|        | 207 | G-A | | Yes | |
|        | 263 | A-G | | Yes | |
| Position | Mutation | Amino Acid Change | Frequency | Present |
|----------|----------|-------------------|-----------|---------|
| 310      | T-C      |                   | Yes       |         |
| 489      | T-C      |                   | Yes       |         |
| 16129    | G-A      |                   | Yes       |         |
| 16189    | T-C      |                   | Yes       |         |
| 16223    | C-T      |                   | Yes       |         |
| 16248    | C-T      |                   | Yes       |         |
| 16297    | T-C      |                   | Yes       |         |
| 750      | A-G      |                   | Yes       |         |
| 1438     | A-G      |                   | Yes       |         |
| 2706     | A-G      |                   | Yes       |         |
| 4071     | C-T      |                   | Yes       |         |
| 4164     | A-G      |                   | Yes       |         |
| 4679     | T-C      |                   | Yes       |         |
| 4769     | A-G      |                   | Yes       |         |
| 5351     | A-G      |                   | Yes       |         |
| 5460     | G-A      | Ala331Thr         | 5.88%     | Yes     |
| 6455     | C-T      |                   | Yes       |         |
| 6680     | T-C      |                   | Yes       |         |
| 7028     | C-T      |                   | Yes       |         |
| 7684     | T-C      |                   | Yes       |         |
| 7853     | G-A      | Val90Ile          | 29.41%    | Yes     |
| 8701     | A-G      | Thr59Ala          | 52.94%    | Yes     |
| 9540     | T-C      |                   | Yes       |         |
| 9824     | T-C      |                   | Yes       |         |
| 10398    | A-G      | Thr114Ala         | 41.18%    | Yes     |
| 10400    | C-T      |                   | Yes       |         |
| 10873    | T-C      |                   | Yes       |         |
| 11719    | G-A      |                   | Yes       |         |
| 12405    | C-T      |                   | Yes       |         |
| 12705    | C-T      |                   | Yes       |         |
| 12811    | T-C      | Tyr159His         | 64.71%    | Yes     |
| 14766    | C-T      | Thr7Ile           | 47.06%    | Yes     |
| 14783    | T-C      |                   | Yes       |         |
| 14978    | A-G      | Ile78Val          | 47.06%    | Yes     |
| SNP   | Change | Alternative | Frequency | Present |
|-------|--------|-------------|-----------|---------|
| 15043 | G-A    |             | Yes       |
| 15301 | G-A    |             | Yes       |
| 15326 | A-G    | Thr194Ala   | 52.94%    | Yes     |
| tRNA^Thr |      |             | 100%      | No      |

As presented in online mitochondrial genome databases: www.mitomap.org and www.genpat.uu.se/mtDB.

**Figures**
Figure 1

The Chinese pedigree with CHD. (A) Vision-impaired individuals are indicated by blackened symbols. (B) Identification of the 15910C>T mutation in the tRNA gene. Partial sequences chromatograms of tRNA gene from the proband and one Chinese control. An arrow indicates the location of the base changes at position 15910.
Figure 2

Northern blot analysis of mitochondrial tRNA. (A) Equal amounts of total mitochondrial RNA from various cell lines were electrophoresed through a denaturing polyacrylamide gel, electroblotted and hybridized with DIG-labeled oligonucleotide probes specific for the tRNAThr, tRNAHis, tRNAGlu, tRNAAla respectively. (B) Quantification of tRNA levels. Average relative tRNA content per cell, was normalized to the average content per cell of 5S rRNA in three mutant cybrid cell lines (III1-3, III1-5 and III1-8) carrying the 15910C>T and control cybrid cell lines (C2-2, C2-8 and C2-9). The values for the latter are expressed as percentages of the average values for the control cell lines.
Western blot analysis of mitochondrial proteins. (A) Twenty micrograms of total cellular proteins from various cell lines were electrophoresed through a denaturing polyacrylamide gel, electroblotted and hybridized with respiratory complex subunits in mutant and control cells with VDAC as a loading control. (B) Quantification of 6 respiratory complex subunits. The levels of ND6, ND4, ATP6, CYTB, CO2 and ND1 in three mutant cybrid cell lines and control cybrid cell lines were determined. The error bars indicate two standard errors of the means. p indicates the significance, according to the t-test, of the differences between mutant and control cell lines.
Measurement of cellular in mitochondria. (A) Respiratory complex activities. The activities of respiratory complexes were investigated by enzymatic assay on complexes I, II, III, and IV in isolated mitochondria from lymphoblastoid cell lines derived from the mutant and control cybrid cell lines. Activities of complexes I, II, III, and IV were normalized by citrate synthase activity. (B) mitochondrial ATP levels. Mutant and control cell lines were incubated with 10 mM glucose or 5 mM 2-deoxy-d-glucose plus 5 mM pyruvate to determine ATP generation under mitochondrial ATP synthesis. Average rates of ATP level per cell line in mitochondria are shown. The determinations were made for each cell line.
calculations were based on the independent determinations in each cell line. (C) Ratio of geometric mean intensity. Measurement of mitoROS. The levels of ROS generation by mitochondria in living cells from mutant and control cell lines were determined using the mitochondrial superoxide indicator MitoSOX-Red. The average of the determinations for each cell line is shown. The error bars indicate two standard errors of the means. p indicates the significance, according to the t-test, of the differences between mutant and control cell lines.