Mitochondrial DNA Mutations in Cardiomyopathy

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Deletions and point mutations of mitochondrial DNA (mtDNA) of patients with dilated or hypertrophic cardiomyopathy were analyzed using the polymerase chain reaction and fluorescence-based direct sequencing. The patients included are with hypertrophic cardiomyopathy associated with left ventricular dilatation, a patient with mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS), and a patient with fatal infantile cardiomyopathy. Deletions were frequently seen in mtDNA in patients with dilated cardiomyopathy. The mtDNA was sequenced and the direct repeat at each edge of deletion was identified as (5'-CATCAACAACC-3') which was located in the ATPase6 gene and in the D-loop region. In a patient with hypertrophic cardiomyopathy associated with left ventricular dilatation, another mutant mtDNA was found not to have directly repeated sequence, and was revealed to jump from nucleotide position 8,992 to position 16,072 of mtDNA resulting in a 7,079 bp deletion. This patient had unique point mutation in the tRNA genes. A G-to-A transition in the tRNA{\textsuperscript{Cys}} gene (nucleotide position 5,821) at the aminoacyl acceptor stem and an A-to-G transition in the tRNA{\textsuperscript{Thr}} gene (nucleotide position 15,951) were identified. In a patient with MELAS, an A-to-G transition in the tRNA{\textsuperscript{Leu(UUR)}} gene (nucleotide position 3,243) was observed. This mutation was located at the 5' end of the dihydrouridine loop of this tRNA molecule, and would disturb its function. In a patient with hypertrophic cardiomyopathy associated with lactic acidosis, mutations of mtDNA should be suspected. In a patient with fatal infantile cardiomyopathy, three point mutations in the genes of tRNA{\textsuperscript{Leu}} (nucleotide position 3,254), tRNA{\textsuperscript{Ile}} (nucleotide position 4,317), and tRNA{\textsuperscript{Trp}} (nucleotide position 5,554) were identified. From these data, it is suggested that mtDNA deletions and point mutations which induce base substitutions in protein subunit genes, and base substitutions in tRNA genes which affect the function of the mitochondrial respiratory chain are important contributory factors to the genesis of some forms of cardiomyopathy.

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ALTHOUGH the cause of cardiomyopathy remain unclear, cardiomyopathy is widely accepted as a multifactorial disease. Various factors such as viral infections, chronic ingestion of excessive alcohol, pregnancy, microvascular hyperreactivity, and free radicals have been proposed to participate in its genesis. Recently the role of DNA mutations has been emphasized as an etiology of familial dilated{\textsuperscript{1}} or hypertrophic cardiomyopathy{\textsuperscript{2}}. Two forms of familial cardiomyopathy are known: X-linked recessive dilated cardiomyopathy{\textsuperscript{1}}; and autosomal

Key words:
- Dilated cardiomyopathy
- Hypertrophic cardiomyopathy
- Mitochondrial DNA
- MELAS syndrome
- Mutation

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TABLE I CHARACTERISTICS OF PATIENTS AND CONTROL

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age (year)</th>
<th>Clinical diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>T.Y.</td>
<td>F</td>
<td>54</td>
</tr>
<tr>
<td>2</td>
<td>K.M.</td>
<td>F</td>
<td>68</td>
</tr>
<tr>
<td>3</td>
<td>K.I.</td>
<td>M</td>
<td>37</td>
</tr>
<tr>
<td>4</td>
<td>K.Y.</td>
<td>M</td>
<td>21</td>
</tr>
<tr>
<td>5</td>
<td>Y.Y.</td>
<td>F</td>
<td>49</td>
</tr>
<tr>
<td>6</td>
<td>T.K.</td>
<td>M</td>
<td>65</td>
</tr>
<tr>
<td>7</td>
<td>Y.M.</td>
<td>M</td>
<td>52</td>
</tr>
<tr>
<td>8</td>
<td>T.S.</td>
<td>F</td>
<td>68</td>
</tr>
<tr>
<td>9</td>
<td>H.O.</td>
<td>M</td>
<td>53</td>
</tr>
<tr>
<td>10</td>
<td>T.N.</td>
<td>M</td>
<td>43</td>
</tr>
<tr>
<td>11</td>
<td>K.S.</td>
<td>M</td>
<td>47</td>
</tr>
<tr>
<td>12</td>
<td>K.S.</td>
<td>M</td>
<td>69</td>
</tr>
<tr>
<td>13</td>
<td>T.K.</td>
<td>F</td>
<td>40</td>
</tr>
<tr>
<td>14</td>
<td>F.S.</td>
<td>M</td>
<td>75</td>
</tr>
<tr>
<td>15</td>
<td>K.M.</td>
<td>M</td>
<td>40</td>
</tr>
<tr>
<td>16</td>
<td>M.H.</td>
<td>F</td>
<td>59</td>
</tr>
<tr>
<td>17</td>
<td>E.S.</td>
<td>F</td>
<td>42</td>
</tr>
</tbody>
</table>

tricular dilatation, (3) a patient with mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) showing hypertrophic cardiomyopathy, and (4) a patient with fatal infantile cardiomyopathy.

MATERIALS AND METHODS

Patients

Study (1): Cardiac tissue specimens were obtained from 16 patients with hypertrophic or dilated cardiomyopathy of unknown etiology and from an accident victim as a normal control. Patient-13 presented with familial dilated cardiomyopathy. Their clinical features are shown in Table I. Autopsied frozen myocardiums of patient-5, 9, and 11 as reported by Ozawa et al9 were used.

Study (2): A 43-year-old man with a twenty year history of cardiomegaly was admitted to hospital because of general malaise and dyspnea on exertion. The chest X ray showed cardiac enlargement with a 68% cardio-thoracic ratio and evidence of pulmonary congestion and bilateral effusion. The echocardiogram showed left ventricular enlargement, diffuse hypokinesis in wall motion. The ejection fraction was 20%. The cardiac index was 2.5 l/min/m². The endomyocardial biopsy samples showed myofiber hypertrophy and marked disarray, which are characteristic of hypertrophic cardiomyopathy, although the hemodynamic features resembled dilated cardiomyopathy. Family history showed that his younger sister also had hypertrophic cardiomyopathy. Two years after the first admission, he died of heart and renal failure. Autopsied frozen heart muscle, skeletal muscle and liver of this patient as reported by Hattori et al10 and Ozawa et al11 were used.

Study (3): A 37-year-old man was admitted to a hospital because of abdominal pain, vomiting, and fainting. He had lactic acidosis, hypertrophic cardiomyopathy, and stroke-like episodes, and died of heart failure. Ragged-red fibers were observed on skeletal muscle biopsy.

Study (4): A 1-year-old boy was admitted to a hospital because of general weakness. He was the only one child of non-consanguineous healthy parents. A chest roent-
TABLE II SYNTHESIZED PRIMERS USED FOR PCR

<table>
<thead>
<tr>
<th>Primers*</th>
<th>Sequence 5'→3'</th>
<th>Complementary site**</th>
</tr>
</thead>
<tbody>
<tr>
<td>L116</td>
<td>AACTCAAAGGACCTGCGGT</td>
<td>1,161 to 1,180</td>
</tr>
<tr>
<td>L288</td>
<td>CTACTATACCTCAAATTGATCC</td>
<td>2,881 to 2,900</td>
</tr>
<tr>
<td>L853</td>
<td>ACGAAAATCTGTTGCGTTCA</td>
<td>8,531 to 8,550</td>
</tr>
<tr>
<td>H38</td>
<td>AAATTGAAATCTGCGTTAGG</td>
<td>400 to 381</td>
</tr>
<tr>
<td>H366</td>
<td>GAGTTTTGATGCTACCCCTGA</td>
<td>3,680 to 3,661</td>
</tr>
<tr>
<td>H617</td>
<td>CCGGGGAAAACGGCCATATCGGG</td>
<td>6,190 to 6,171</td>
</tr>
<tr>
<td>FL853</td>
<td>tgaacagcgacgagcagt</td>
<td>8,531 to 8,550</td>
</tr>
<tr>
<td></td>
<td>ACGAAAATCTGCGATTCGA</td>
<td></td>
</tr>
</tbody>
</table>

*The first letter of the primer, L or H, specifies its priming strand.
The addition of letter F represents the primer for fluorescence-based direct sequencing.
**Numbering of the mtDNA is done according to Anderson and colleagues. Lower case letter correspond to the M13 universal sequence.

A genogram revealed severe cardiomegaly with a cardiothoracic ratio of 71%. Cyanosis and stiffness of neck were found. Blood analysis indicated anemia, metabolic acidosis, and increased levels of muscle enzymes (glutamic-oxaloacetic transaminase, glutamic-pyruvic transaminase, lactate dehydrogenase, creatine kinase). The patient developed bradycardia and convulsions, and died of cardiac failure seven days after admission. Frozen autopsied myocardium of this patient as reported by Tanaka et al.12,13 was used.

**Preparation of DNA**

The heart muscles (5 mg per sample) were homogenized with a Physcotron handy microhomogenizer (Niti-on, Tokyo, Japan) for 30 seconds and then digested in 1 ml of 10 mM Tris-HCl, 0.1 M EDTA (pH 7.4) that contained 0.1 mg/ml proteinase K and 0.5% SDS. DNA was extracted twice with equal volumes of phenol/chloroform/isoamyl alcohol (25: 25: 1) and then once with chloroform/isoamyl alcohol (25: 1). DNA was precipitated with a one-fifteenth volume of 5 M NaCl and two volumes of ethanol at −80 °C for 2 h, and then rinsed with 70% ethanol. The precipitated DNA was recovered in 30 μl of 10 mM Tris-HCl, 0.1 mM EDTA (pH 8.0).

**Oligonucleotide primers**

Primers used for polymerase chain reaction (PCR) were synthesized using a Shimadzu model NS-1 DNA synthesizer (Shimadzu Corp, Kyoto, Japan) and an Applied Biosystems model 381A DNA synthesizer (Applied Biosystems Inc, Foster City, CA, USA) and then purified on oligonucleotide purification cartridges (Applied Biosystems) according to the manufacturer’s instruction. For asymmetric PCR amplification to produce a template for the fluorescence-based automated DNA sequence, we used limiting primers with the −21M13 sequence at its 5’ terminus, which were also synthesized and purified as above. The base sequences of the oligonucleotide primers are shown in Table II.

**PCR amplification**

The extracted DNA solution (1 μl) was amplified in 50 μl of reaction mixture containing 200 μM of each dNTP (deoxyribonucleotide triphosphate), 1 μM of each primer, 1.25 units of Taq DNA polymerase (Promega, Madison, WI, USA) and PCR buffer (50 mM Tris-HCl, pH 8.4, containing 50 mM KCl, 1.5 mM MgCl₂ and 0.01% gelatin). The reaction was carried out for a total of 30 amplification cycles with the use of a Perkin-Elmer/Cetus Thermal Cycler (Perkin-Elmer/Cetus Corp., Norwalk, CT, USA). The cycle time was as follows: denaturation, 15 sec at 94 °C; annealing, 15 sec at 50 °C; and primer extension, 80 sec at 72 °C. Amplified fragments were separated by electrophoresis on 1% agarose gel and were detected after they had been stained with ethidium bromide.

**Southern blot analysis**

Total DNA (100 ng) was digested with 12 units of PvuII and PstI obtained from

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Fig. 1. A: Detection of the deleted mtDNA in hearts of patients with cardiomyopathy using the PCR method. The mtDNA fragments were amplified using primers L853 and H38, separated on a 1% agarose gel, and stained with ethidium bromide. Lanes are arranged in accordance with Table I. Sizes of amplified fragments are indicated in kb.

B: PCR amplification using primers L116 and H617, the distance between them is 5.0 kb. The normal-sized fragments of 5.0-kb are amplified and the amounts of the fragments are approximately the same in all patients.

DNA digested with HaeIII from Nippon Gene (Toyama, Japan). DNA in the gels was denatured and transferred onto Hybond-N+ membrane from Amersham, UK. Hybridization using the PCR-amplified mtDNA fragments as the probes was carried out with the Enhanced Chemiluminescence Gene Detection System (ECL kit) from Amersham, UK.

Asymmetric PCR amplification for template formation of Fluorescence-based sequencing

Asymmetric amplification was carried out on 1 μl of the primary PCR product in a final volume of 50 μl which included the reagents described with 0.5 pmol of limiting primers with the -21M13 sequence at its 5' terminus (Table II) and 50 pmol of H primer with 1.25 units of Taq DNA polymerase. PCR was performed for a total of 30 cycles as above. The PCR product containing single-stranded DNA was precipitated with 5 μl of sodium acetate (pH 7.4) and 120 μl of ethanol. After incubation at -20°C for 6 h, the mixture was centrifuged at 11,000×g for 10 min.

Toyobo (Osaka, Japan), and separated electrophoretically on 0.6% agarose gels. Size standards employed were lambda phage DNA digested with HindIII and phage X174 DNA.
The pellet was rinsed with 120 \( \mu l \) of 70% ethanol, dried in a vacuum for 15 min, and dissolved in 8 \( \mu l \) of distilled water.

**Fluorescence-based direct sequencing**

Dye primers and a Taq Sequencing Kit were obtained from Applied Biosystems. For the sequencing reactions with Joe dye primer (for adenine; A) and the Fam dye primer (for cytosine; C), the following reagents were mixed to a total volume of 7.75 \( \mu l \): 1.0 \( \mu l \) of single-stranded DNA template, 1.0 \( \mu l \) of each dye primer (dissolved to a concentration of 0.4 pmol/\( \mu l \)), 1.0 \( \mu l \) of the 5 \( \times \) Taq sequencing buffer (50 mM Tris-HCl, pH 8.5, 50 mM MgCl\(_2\), 250 mM NaCl), 1.0 \( \mu l \) of each mixture of d/ddNTP (deoxyribonucleoside/dideoxyribonucleoside triphosphate) and 3.5 \( \mu l \) of distilled water, and 0.25 \( \mu l \) of Taq DNA polymerase (5 U/\( \mu l \); Promega). For the sequencing reactions with Tmra dye primers (for guanine; G) and Rox dye primers (for thymine; T), the amounts of reagents were doubled to a total volume of 15.5 \( \mu l \). Ten cycles of denaturation at 90°C for 15 sec, annealing and extension at 70°C for 60 sec were conducted in the Thermal Cycler. The contents of the four tubes were pooled into one tube containing a mixture of 5.0 \( \mu l \) of sodium acetate (pH 5.2) and 120 \( \mu l \) of ethanol. After incubation at -20°C for 10 min, the mixture was centrifuged at 11,000 \( \times g \) for 10 min. The pellet was rinsed with 100 \( \mu l \) of 70% ethanol, and dried in a vacuum chamber for 10 min and stored at -20°C. Just prior to electrophoresis, the pellet was dissolved in the mixture of 5 \( \mu l \) of deionized formamide and 1 \( \mu l \) of 50 mM EDTA (pH 8.0). The DNA samples were denatured by heating at 90°C for 2 min, immediately cooled on ice, and then loaded onto 6% acrylamide gel. Fluorescence-based automated DNA sequence analysis was carried out using a 373A DNA Sequencer (Applied Biosystems Inc.) run with the manufacture’s version of 1.0.1 software.

**Digestion of amplified mtDNA fragment with Apal**

mtDNA was amplified by PCR, using primers L288-H366. The amplified fragment was digested with ApaI and separated on a 1% agarose gel, and stained with ethidium bromide.

**RESULTS**

Study (1): Fig.1-A shows the PCR amplification of mtDNA from the heart muscle using primers L853 and H38. Multiple abnormal bands including a 1.0-kb fragment, which means a 7.4 kb-deletion of mtDNA, was detected in patients 5, 6,
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Table III: Enzyme Activities in Heart Mitochondria

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Patient (%)</th>
<th>Control* (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complex I</td>
<td>213 (31)</td>
<td>690 ± 267 (3)</td>
</tr>
<tr>
<td>Complex II</td>
<td>1866 (309)</td>
<td>604 ± 98 (3)</td>
</tr>
<tr>
<td>Complex III</td>
<td>1119 (110)</td>
<td>1017 ± 696 (6)</td>
</tr>
<tr>
<td>Complex IV</td>
<td>197 (33)</td>
<td>593 ± 122 (3)</td>
</tr>
<tr>
<td>Complex V</td>
<td>106 (80)</td>
<td>133 ± 42 (3)</td>
</tr>
</tbody>
</table>

Activities are expressed in nmol/min/mg of mitochondrial protein.
*Values are mean ± SD.

The Southern blot analysis revealed only the 16.6-kb fragment after digestion with PvuII. When PsI was used as the restriction enzyme, only two fragments of 14.5 kb and 2.1 kb were observed. No abnormal fragments were detected by the conventional Southern blot method in the patient’s heart muscles.

The deletion was confirmed by the primer shift PCR method and PCR Southern method. These results indicate that the PCR products observed here are not artifacts but are amplified from the deleted mtDNA.

Sequence of the region surrounding the deletion is shown in Fig. 2. The crossover sequence was demonstrated to be a 12-bp directly repeated sequence of 5'-CATCAAACCCGT-3', which was located at the boundaries of the deletion between the ATPase6 gene and the D-loop region. The deletion spanned 7,436 bp from position 8,649 to position 16,084.

Study (2): Fig. 3 shows the PCR amplification of mtDNA from the heart muscle, skeletal muscle, and liver of patient using primer L853 and H38. Multiple abnormal fragments (3.1 kb, 2.4 kb, 2.3 kb, 2.0 kb, 1.7 kb, 1.4 kb, and 1.0 kb), which mean 5.3 kb, 6.0 kb, 6.1 kb, 6.4 kb, 6.7 kb, 7.0 kb and 7.4 kb-deletions of mtDNA respectively, were detected. The sizes and the amounts of deletions were different among these organs. The crossover sequence of one mutant mtDNA was demonstrated to be a 12-bp directly repeated sequence of 5'-CATCAAACCCGT-3', which was located in the crossover point of deletion between the ATPase6 gene and the D-loop region. The deletion spanned 7,436 bp form position
8,649 to position 16,084. The deletion was the same as that in Study 1. Another mutant mtDNA was demonstrated not to have directly repeated sequence, and was revealed to jump from position 8,992 to position 16,072 of mtDNA resulting in a 7,079 bp deletion. This patient had two unique point mutations in the tRNA genes. A G-to-A transition at nucleotide position 5,821 in the tRNA_Cys gene at the aminoacyl acceptor stem and an A-to-G transition at position 15,951 in the tRNA Thr gene were found. Both transitions would decrease the stability of the tRNA acceptor stem.

Study (3): We identified an A-to-G transition at 3,243 in the tRNA_{Leu(UUR)} gene (Fig. 4) in a patient with MELAS by fluorescence-based automated direct sequencing. This mutation located at the 5' end of the dihydrouridine (DHU) loop of this tRNA molecule (Fig. 5). This mutation was observed in another MELAS patient and was absent in controls. This mutation creates an ApaI restriction site. The A at position 3,243 in the dihydrouridine loop of the tRNA molecule is strictly conserved in the mtDNA sequences in bovine, mouse, rat, chicken, Xenopus laevis, sea urchin, and Drosophila yakuba, and an A-to-G transition would disturb the function of leucine tRNA.

Study (4): The respiratory enzyme activity of isolated heart mitochondria were analyzed. The activities of Complex I and Complex IV were decreased to 31% and 33% of control values, respectively (Table III). The activity of Complex II was 309% of control, indicating selective defects in Complex I and IV. We sequenced the mtDNA from the patient’s heart. We found several nucleotide transitions which would cause substitutions of amino acids that are conserved among species. One point mutation at nucleotide position 7,673 in the COII gene altered isoleucine to valine. We also found an A-to-G base transition at nucleotide position 4,317 in the tRNA_{Ile} gene. Calculation of the most stable configuration of the tRNA_{Ile} molecule indicated that this mutation add a G=C base pair in the TΨC loop and alters base pairing in the TΨC stem (Fig. 6). We also found a C-to-T transition at nucleotide position 3,254 in the tRNA_{Leu} gene (the D stem), and a C-to-T transition at

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nucleotide position 5,554 in the tRNA^{Trp} gene (the variable loop region).

DISCUSSION

Apart from the minor contribution of anaerobic glycolysis, mitochondria produce adenosine triphosphate exclusively and mitochondrial function is closely related to maintenance of cellular integrity. Several subunits of the mitochondrial electron transport chain are biosynthesized from the information of mtDNA. Complex I (NADH-ubiquinone oxidoreductase), Complex II (succinate-ubiquinone oxidoreductase), Complex III (ubiquinol-cytochrome c oxidoreductase), Complex IV (cytochrome c oxidase), and Complex V (F_{0}F_{1}-ATPase) consist of 25, 4, 10, 7, and 12 subunits, respectively.\(^{15}\) Mitochondrial DNA codes for 7 of 25 subunits in Complex I, 3 of 7 in Complex IV, 2 of 12 in Complex V, and 1 of 10 in Complex III, and these subunits are synthesized in mitochondria.\(^{4,16}\) The other subunits are coded by nuclear DNA, synthesized in cytoplasm, and transferred to mitochondria. It is well known that incidence of mtDNA mutation is ten times higher than that of nuclear DNA mutation\(^{5}\) and that mtDNA mutation is maternally inherited.\(^{7,17}\) It has also been revealed that acquired mtDNA mutations occur among patients without a family history of mutations.\(^{14,18}\) Hence, it is assumed that mtDNA mutations are the responsible factor for mitochondrial dysfunction derived from genetic and/or acquired impairment. mtDNA with a 7.4 kb deletion frequently existed among specimens from patients with dilated cardiomyopathy with or without family history. These results suggest that heart mtDNA deletions may contribute to accelerate pump failure in patients with dilated cardiomyopathy.

In a patient with hypertrophic cardiomyopathy associated with left ventricular dilatation, we found at least five types of deletions of heart mtDNA and two point mutations in tRNA genes. In the presence of these deleted and mutant mtDNA, the integrity of the electron transport chain would be destroyed, resulting in significant impairment of adenosine triphosphate production and consequent heart failure. The ratio of mtDNA to mitochondrion was essentially constant in all cell types, i.e. 2–3 mtDNA molecules/mitochondrion\(^{19}\) and several thousands copies of mtDNA are considered to exist in a cell. In this patient, we detected mtDNA mutations in heart, skeletal muscle and liver. The mutations differed both qualitatively and quantitatively from organ to organ. We could hardly detect mtDNA mutations in platelets. Accordingly, the different amounts of deleted mtDNA in the various tissues might be due to unequal distribution of the altered mtDNA during cell proliferation.

MELAS is a distinctive clinical syndrome characterized by short stature, seizures, and hemiparesis, hemianopsia, or cortical blindness.\(^{6,20}\) Cardiomyopathy is one of the major causes of death in MELAS. Recent studies have demonstrated that MELAS is caused by a deficiency in Complex I of the mitochondrial respiratory chain.\(^{21}\) We have identified mtDNA mutations especially a transition of phylogenetically conserved A-to-G at the 5' end of the DHU loop of tRNA^{Leu} (UUR) that was common among MELAS patients.\(^{13}\) Fifty-six leucine residues encoded by UUR are present in the subunit of Complex I. This abundance of leucine residues in Complex I might explain the apparently selective deficiency of Complex I in MELAS. These results indicate that mtDNA mutations especially the A-to-G transition in the tRNA^{Leu} gene is related to MELAS. This A-to-G transition creates an Apal restriction site, because this enzyme cleaves the mutant sequence (GGGGCC) but not the wild-type sequence (GAGCCC). This convenient method is useful for genetic diagnosis of MELAS. In patients with hypertrophic cardiomyopathy accompanied by lactic acidosis, genetic analysis of mtDNA is recommended.

In a case of fatal infantile cardiomyopathy, we found three point mutations in the genes of tRNA^{Leu} (nucleotide position 3254), tRNA^{Ile} (nucleotide position 4317) and tRNA^{Trp} (nucleotide position 5554). Calculation of the most stable configuration of the tRNA^{Ile} molecule indicated that this mutation adds a G=C base pair in the TΨC loop and alters base pairing in the TΨC stem.\(^{12}\) It is suggested point mutations in the genes of tRNAs in mtDNA might cause

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severe dysfunction of the respiratory chain and might cause early development of heart failure.

In conclusion, it is suggested that mtDNA deletions and point mutations which induce base substitutions in protein subunit genes, and base substitutions in tRNA genes, are important contributory factors to the genesis of some forms of cardiomyopathy.

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