Identification of SNPs in the Coding Region of Human mtDNA

Cassidy Punt
Minnesota State University, Mankato

Elizabeth Smalley
Minnesota State University, Mankato

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Identification of Single Nucleotide Polymorphisms in the Coding Region of Human Mitochondrial DNA

Cassidy L. Punt (Chemistry & Geology)  
Elizabeth Smalley (Chemistry & Geology)  
Dr. Theresa Salerno, Faculty Mentor (Chemistry & Geology)

ABSTRACT

Two novel single nucleotide polymorphisms (SNPs) were discovered within the coding region of the NADH dehydrogenase subunit 2 gene of mitochondrial DNA (mtDNA). mtDNA is of particular importance in forensic analysis as well as in the study of the origin and dispersal of humans. Two segments of the coding region of human mtDNA, as well as the hyper-variable region 2 (HV 2) were selected and sequenced in order to determine if any previously unknown SNPs were present in our test subjects. Target regions were designed to include known SNPs; appropriate primers were developed using the OLG0 6 Primer Analysis Software. The DNA was isolated using the QIAGEN® Generation Capture Column DNA Extraction kit and target regions were amplified via polymerase chain reaction (PCR). Once the fragment sizes were verified using acrylamide gel electrophoresis, the template DNA was prepared for sequencing by PCR using forward primers and IRDye™ labeled dideoxy-terminators. Sequencing PCR products were then purified to remove excess primers and nucleotides and sequenced with the LICOR NEN model 4300 slab-gel DNA sequencer. The SNP analyses developed in this research have been implemented into the Minnesota State University, Mankato biochemistry laboratory curriculum. The two novel SNPs identified were: T644G and T4733G inside the NADH dehydrogenase subunit 2 gene. Each of these occurred in only one of our test subjects. No novel SNPs were found in the HV 2 region. However, sequencing was successful for only one of DNA test samples. Seven previously known SNPs were also found: G3010A and C3116T contained in the 16S rRNA coding region; T4216C contained in the NADH dehydrogenase 1 subunit gene; C4312T contained in the isoleucine tRNA gene; G4491A, T4586C, and T4688C contained in the NADH dehydrogenase 2 subunit gene.
INTRODUCTION

Human mitochondrial DNA (mtDNA) is a circular double-stranded DNA molecule composed of 16568 base pairs. Multiple copies of mtDNA are found in varying numbers in the matrix of eukaryotic mitochondria. The mitochondrial genome codes for 22 tRNAs, 2 rRNAs, and 13 polypeptides which are involved in the electron transport chain.

In addition to the coding regions, mtDNA has a control region of approximately 1000 bases. It contains the origin of replication as well as the bidirectional promoter for both heavy and light strand transcription. This promoter is therefore responsible for the transcription of all RNAs in the mitochondria. This is made possible by the fact that the entire mtDNA genome is transcribed at once. The large RNA molecules produced are then post-transcriptionally processed into mRNAs, tRNAs, and the two rRNAs.

In mammals, mtDNA is maternally inherited due to the relatively high number of copies contributed by the female ovum during fertilization. For this reason, mtDNA analysis is commonly used for maternity testing and is useful for forensic and anthropological studies.

Mitochondria contain the electron transport chain and are, therefore, responsible for most of the adenosine-triphosphate (ATP) production in eukaryotic cells. Mutations of mtDNA are commonly caused by oxygen radicals that are produced during oxidative phosphorylation. The close proximity of mtDNA to the processes of oxidative phosphorylation in the mitochondrial matrix and the absence of a protective membrane cause mtDNA to undergo mutation 5 to 10 times faster than nuclear DNA [Luo, 2007]. mtDNA mutations commonly manifest as single nucleotide polymorphisms (SNPs), which are DNA sequence variations of a single nucleotide between individuals of the same species. These relatively high mutation rates enable the comparison of individuals within a species as well as between closely related species. Some of these SNPs have been associated with specific muscular and neuro-degenerative diseases including those listed in Appendix Table 1A [Chinnery, 2008]. Therefore, these comparisons are widely used in the study and diagnosis of certain metabolic diseases in addition to forensic and archaeological DNA analysis, and the study of human evolution and dispersion. These analyses are aided by the ability of mtDNA to survive in skeletal and dental remains due to its more protected location inside the mitochondrial double membrane.

In the last 10 years, there have been several studies which have focused on the understanding of the evolution of the mitochondrial genome. Ingman and co-workers [2000] studied the genomes of 53 different individuals who represented a diversity of ethnic groups. From their work and the work of many others, several databases have been developed, including the Human Mitochondrial Genome.
These studies showed that there are two non-coding regions with many SNPs; these are called hyper-variable region 1 (HV 1) at nucleotide positions 16024 – 16365 and hyper-variable region 2 (HV 2) at nucleotide positions 73 – 340 [Kline, 2005]. Forensic analyses were originally dependent on identification of key SNPs in these regions. However, since there was a 1/200 chance of two suspects sharing the same profile, this method was not found to be sufficient for common forensic analysis [Divine, 2005]. Therefore, researchers developed new, more selective analyses using the coding regions.

Haplotypes have also been identified and organized into phylogenic trees. For Western European populations, seven lineages have been named on the basis of SNPs. These have been called H, J, K, T, U, V, and X after the so-called “daughters of Eve” with full names of Helena, Jasmine, Katrina, Tara, Ursula, Velma, and Xenia. These haplotypes were originally designated on the basis of SNPs found in the hypervariable regions. For example, the J haplotype was identified using the 16069T, 16126C and the 295T [Álvarez-Iglesias, 2007]. This method, however, was found to be too restrictive for the sub-typing of haplotype groups. Therefore, analyses of coding region SNPs were developed [Quintáns, 2004]. These have been recently applied to populations other than the Western European group. These populations have different lineage designations which have resulted in other trees like the one found in Appendix Figure 1A which describes East Asian and Native American haplogroups [Álvarez-Iglesias, 2007].

There are many methods that are commonly used for SNP analyses. The first, complete genome sequencing, is very useful for the analysis of haplogroups used in the study of human evolution and dispersion as well as identification of SNPs that can be specifically linked to disease. However, this method is tedious, time consuming, and too expensive to utilize for routine forensic casework and disease diagnosis. Therefore, other methods have been developed for these uses. SNaPshot minisequencing is a method that utilizes single base pair extension with allele-specific primers in order to identify the presence or absence of particular SNPs. These reactions are commonly combined to analyze multiple SNPs at once [Álvarez-Iglesias, 2007; Parson, 2007]. The advantage of minisequencing is that it can be targeted to short DNA sequences and therefore, it can be used to analyze degraded DNA. The high throughput of this method makes it an important screening tool for multiple samples.

Another method which allows the analysis of multiple SNPs is single-strand conformation polymorphism (SSCP). This method is commonly used to detect ABO genotypes for forensic testing and could be used for the detection of other SNPs including those of mtDNA. SSCP is performed by the amplification of specific regions of a gene sequence followed by denaturation and electrophoresis. After
denaturation, the single-strand DNA sequences take on a 3-dimensional conformation that can differ according to DNA sequence variations. These different conformations can be separated by gel electrophoresis and subsequently identified [Honda, 2004].

More recently, microarray-based DNA sequencing methods have been developed which are useful as screening tools for known mitochondrial SNPs [Carr, 2008]. In this technique the genomic DNA is labeled with a fluorescent tag and is presented to four different oligonucleotide probes that are bound to a glass or nylon substrate. Each of the four probes varies only in the 3’ terminal position, each having one of the four nucleotides. Therefore, the tagged genomic DNA will bind only to the probe whose 3’ terminus corresponds to the nucleotide, or SNP, in question. This bound DNA is then detected using computerized scanners which detect the fluorescent tags.

DESIGN

The objectives of our research were to search for previously reported high frequency SNPs as well as reveal any new SNPs existing within selected coding regions of mtDNA and a section of the hypervariable region. In order to accomplish this we needed to design and optimize the polymerase chain reaction (PCR) conditions for both the target sequence amplification and the DNA sequencing reactions. Initially, we targeted four regions of the human mitochondrial genome shown in Table 1 and Figure 1 on the following page. Three of the target regions were contained in the coding region of the mtDNA and were selected because of the frequencies of reported SNPs [Quintáns, 2004; Brandstätter, 2004]. The fourth target region was designed to encompass the noncoding control region HV 2. HV 2 was selected due to its common use in early studies of human evolution and dispersion [Salas, 2000]. Primers were designed using OLIGO 6 Primer Analysis Software along with the reference sequence: GenBank accession number AC000021. Each of the target regions was designed to cover approximately 700 base pairs, which was the expected limit of detection in the DNA sequencing analysis.
Table 1. Primers used for target region amplification and DNA sequencing, area of mtDNA genome amplified, genes contained, and known SNPs at time of experimental design

<table>
<thead>
<tr>
<th>Primers</th>
<th>Region Amplified</th>
<th>Tm</th>
<th>Genes Contained</th>
<th>Known SNPs</th>
</tr>
</thead>
</table>
| A<sub>F</sub> 5′−CGATTCCGCTACGACCAACT−3′  
A<sub>R</sub> 5′−GCAGGCCGGATGTACG−3′ | 4141 - 4839 | 65°C | 4141-4265 NADH DH-1<sup>p</sup>  
4266-4335 Ile tRNA  
4336-4405 Gln tRNA  
4406-4468 Met tRNA  
4469-4839 NADH DH-2<sup>p</sup> | T4216C  
T4646C |
| B<sub>F</sub> 5′−CTACCAGCTCGGAATAA−3′  
B<sub>R</sub> 5′−GGGCATCCATATAGTCACT−3′ | 6645 - 7380 | 59°C | Cyt C Oxidase 1<sup>p</sup> | C6776T  
T7028C |
| C<sub>F</sub> 5′−GAGGCCGCCGCAAACA−3′  
C<sub>R</sub> 5′−GGGCCTTTGCGTAGTTGTAT−3′ | 2695 - 3397 | 63°C | 2695-3234 16S rRNA<sup>d</sup>  
3235-3304 Leu tRNA  
3307-3397 NADH DH-1<sup>p</sup> | G3010A  
C3116T |
| HV 2<sub>F</sub> 5′−ACTCTAGGCCAGACCT−3′  
HV 2<sub>R</sub> 5′−CCTCTGTGCCTGGATATT−3′ | 15724 - 16419 | 60°C | Non-Coding Region |

*<sup>p</sup> denotes partial inclusion of gene

Figure 1. mtDNA genome map with corresponding target regions

Map adapted from Ruiz-Pesini, 2007
METHODS

Whole DNA was extracted from blood samples using the QIAGEN® Generation Capture Column DNA extraction kit. The development of the methods was done with the researchers’ DNA samples. Subsequent analyses were performed by individual students in the Minnesota State University, Mankato biochemistry laboratory classes. Individual PCR reactions for each DNA sample were prepared using designed primers under the following conditions: 20nM template DNA, 1X PCR buffer, 2.0mM Mg²⁺, 0.2mM ea. dNTPs, 0.5 μM ea. primers, 0.02 Units/μL Taq polymerase. Two separate temperature programs were performed for Regions A & C, and B & HV 2 due to large differences in melting temperature of the corresponding primers. After a 95°C pre-incubation step for 5 min, PCR was performed for a total of 30 cycles under the following conditions: 59/55°C annealing for 1 min, 70°C extension for 2 min, 95°C denaturation for 1 min [adapted from Coene, 2004; Kline, 2005; Quintáns, 2004]. PCR products were then purified using the MinElute® PCR Purification Kit (Qiagen) to remove unincorporated primers and nucleotides, and finally analyzed using 5% polyacrylamide gel electrophoresis (BIO-Rad Criterion gels) and silver stained (GE Healthcare kit) to ensure the presence of PCR products of the correct size.

Sequencing PCR reactions were then prepared using the LI-COR® IRDYE™ 800 Terminator Sequencing Protocol with a final primer concentration of 0.086 μM. Each set of sample tubes was subjected to a 2 min pre-incubation step at 95°C, followed by 30 cycles under the following conditions: 95°C denaturation for 30s, 59/55°C annealing for 30s, 72°C extension for 45s. PCR reactions were then held at 4°C until being removed from the thermal cycler. Sequencing reaction products were then purified using the Ethanol Precipitation Protocol supplied by LI-COR to remove excess nucleotides.

Purified sequencing reaction products were then electrophoresed on a 66cm 3.7% KB Plus Gel overnight with a 0.8X TBE running buffer to produce sample images for each DNA sample. DNA sequences were then obtained from sample images using LI-COR e-Seq™ DNA Analysis Software and aligned using the UVa FASTA Sequence Comparison Server (http://fasta.bioch.virginia.edu/) and SNPs were determined by comparison with the rCRS mitochondrial sequence found in Mitomap (http://www.mitomap.org/).

RESULTS

Amplified target DNA fragment sizes were verified as having sizes of approximately 700 base pairs (Figure 2); however, amplification PCR reactions were most successful for Regions A and C. Using the initial three DNA samples, amplification and sequencing reactions were less successful for Region B because of inconsistencies in the amount of amplification among the samples and for the HV 2 Region
because of variability in the size of the PCR products obtained.

**Figure 2.** PCR target amplification size analysis

Sequencing attempts of the HV 2 region were not successful for two of the sample DNAs. However, the three purified PCR products of Region C were sequenced successfully and gave clear sample images as shown in **Figure 3A**. A faint lane in the third sample could be analyzed by altering intensities. Two SNPs were found for two different DNA samples. Both of these, G3010A and C3116T were found in the 16S rRNA gene.

**Figure 3A.** Sample image

**Figure 3B.** Comparison of sequencing analyses

DNA 1 contains the SNP: G3010A

The initial three PCR amplifications of Region A were also successfully sequenced. Later analysis by biochemistry lab students gave a combined total of 12 mitochondrial DNA samples. Seven SNPs were
found in these samples representing region A. They included: T4216C in the NADH dehydrogenase 1 subunit gene; C4312T in the isoleucine tRNA gene; G4491A, T4586C, and T4688C in the NADH dehydrogenase 2 subunit gene [Ruiz-Pesini, 2007]. The two novel SNPs identified were T4644G and T4733G inside the NADH dehydrogenase 2 subunit gene. Both of these previously unreported SNPs were found in only one of the tested DNA samples. A summary of the SNPs found in this research is given in Table 2.

**Table 2.** SNPs identified by region with frequency, associated mutation, and haplogroup

<table>
<thead>
<tr>
<th>Region</th>
<th>SNP Found</th>
<th>SNP Frequency</th>
<th>Gene Location &amp; Δ Amino Acid</th>
<th>Haplogroup Association</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>T4216C</td>
<td>9.00%</td>
<td>Europe ND 1 / Tyr - His</td>
<td>R Branch Point</td>
</tr>
<tr>
<td></td>
<td>C4312T</td>
<td>1.30%</td>
<td>Africa Ile tRNA</td>
<td>L0 Branch Point</td>
</tr>
<tr>
<td></td>
<td>G4491A</td>
<td>0.70%</td>
<td>Asia ND 2 / Val - Ile</td>
<td>Haplogroup M9</td>
</tr>
<tr>
<td></td>
<td>T4586C</td>
<td>1.00%</td>
<td>Africa ND 2 / Syn</td>
<td>Haplogroup L0</td>
</tr>
<tr>
<td></td>
<td>T4644G</td>
<td>No Data</td>
<td>ND 2 / Tyr - Asp</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>T4688C</td>
<td>0.90%</td>
<td>Africa ND 2 / Syn</td>
<td>Haplogroup L3a</td>
</tr>
<tr>
<td></td>
<td>A4731G</td>
<td>No Data</td>
<td>ND 2 / Asn - Lys</td>
<td>None</td>
</tr>
<tr>
<td>C</td>
<td>G3010A</td>
<td>20.30%</td>
<td>E &amp; Asia 16S rRNA</td>
<td>Haplogroup J1</td>
</tr>
<tr>
<td></td>
<td>C3116T</td>
<td>0.30%</td>
<td>Europe 16S rRNA</td>
<td>None</td>
</tr>
</tbody>
</table>

Frequency data and gene location from Ingman, 2006
Haplogroup association adapted from Ruiz-Pesini, 2007
Syn – synonymous (no change in primary structure)

**DISCUSSION**

The amplification PCR protocol developed was successful for the amplification of the targeted regions of the mtDNA, producing DNA fragments of approximately 700 base pairs. However, the PCR product concentrations for Regions B and HV 2 were more variable among the three samples indicating that the amplification did not perform consistently for these regions (Figure 2). This difference in amplified DNA concentration can most likely be attributed to a sequence variation contained within one or both of the priming locations for both Regions B and HV 2. This hypothesis was confirmed in our subsequent sequencing of the PCR products for Regions B and HV 2 as sequencing of these DNA samples did not produce sufficient sample images to attain proper DNA sequences for the DNA samples. Only one short DNA sequence was determined for the HV 2 Region and this sequence was synonymous with the rCRS; i.e., it did not reveal any new SNPs.

The sequencing of DNA samples for Regions A and C, 12 and 3 samples, respectively, revealed seven previously reported SNPs which cause various changes in their respective proteins or tRNAs (Table
None have been previously associated with mitochondrial diseases. Both novel SNPs were found in one of our test subjects of Asian descent (Table 3).

**Table 3. SNPs identified by sample**

<table>
<thead>
<tr>
<th>SNP</th>
<th>Haplogroup Region</th>
<th>Subject Genealogy</th>
</tr>
</thead>
<tbody>
<tr>
<td>T4216C</td>
<td>Europe</td>
<td>American Caucasian</td>
</tr>
<tr>
<td>C4312T</td>
<td>Africa</td>
<td>African</td>
</tr>
<tr>
<td>G4491A</td>
<td>Asia</td>
<td>Asian</td>
</tr>
<tr>
<td>T4586C</td>
<td>Africa</td>
<td>African</td>
</tr>
<tr>
<td>T4644G</td>
<td>Asia</td>
<td>Asian</td>
</tr>
<tr>
<td>T4688C</td>
<td>Africa</td>
<td>American Caucasian</td>
</tr>
<tr>
<td>A4731G</td>
<td>Asia</td>
<td></td>
</tr>
<tr>
<td>G3010A</td>
<td>Europe &amp; Asia</td>
<td>American Caucasian</td>
</tr>
<tr>
<td>C3116T</td>
<td>Widely Distributed</td>
<td>American Caucasian</td>
</tr>
</tbody>
</table>

Haplogroup region adapted from Ruiz-Pesini, 2007

Shaded SNPs were found within the same DNA sample

Using a simplified mitochondrial DNA tree, six of the seven known SNPs have been used to help identify haplotype subgroups in Figure 4 on the following page. Shown in Table 2, the previously reported SNPs were found in varying frequencies among different populations. The frequencies reported are that of 2704 mitochondrial sequences that have been compiled by Ingman and co-workers [2006]. Most of these SNPs are found almost exclusively in the populations indicated by Table 2 and Table 3. However, there are some SNPs which are exceptions. For example, G3010A is commonly found in many different populations. Of the 20.30% frequency in the total number of sequences obtained, this SNP occurs in varying degrees among different genealogies: 2.0% - AfricaN, 0.9% - North American Native, 1.5% South American Native, 46.5% Asian, 0.5% Australian, 44.0% European, 4.6% South Asian. Therefore, some SNPs are associated with multiple haplogroups and sub-types within the complete mitochondrial DNA tree which can be viewed at: [http://www.mitomap.org/mitomap-phylogeny.pdf](http://www.mitomap.org/mitomap-phylogeny.pdf) [Ruiz-Pesini, 2007]. Shown in Table 3 above, all SNPs correspond to their expected haplogroups with the exception of T4688C, which is associated with a specific point in the African mitochondrial tree. However, it is actually found in the sequences of many different populations [Ingman, 2006, Ruiz-Pesini, 2007].
The two novel SNPs, T4644G and T4733G both result in missense mutations of the NADH dehydrogenase 2 subunit protein causing amino acid changes of tyrosine → aspartic acid and asparagine → lysine, respectively, as shown in Figure 5 below.

Figure 5. Novel SNPs and corresponding amino acid changes
ACKNOWLEDGEMENTS

This work was sponsored by grants from the Center for Excellence in Teaching and Learning (CETL) at Minnesota State University, Mankato and the Genomics Education Matching Fund Program from LI-COR® Biosciences. The authors would also like to acknowledge the contributions of Dr. Jim Rife and the Minnesota State University, Mankato biochemistry classes of 2005 and 2007.

REFERENCES

APPENDIX

Table 1A. Genetic classification of some mitochondrial disorders caused by various SNPs

<table>
<thead>
<tr>
<th>Clinical</th>
<th>Specific Symptoms</th>
<th>Gene</th>
<th>Associated Mutation(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leber Hereditary Optic Neuropathy</td>
<td>Optical nerve &amp; retinal degeneration</td>
<td>NADH D4, NADH D6, NADH D1</td>
<td>G11778A, T14484C, G3460A</td>
</tr>
<tr>
<td>Leigh Syndrome</td>
<td>Loss of muscle control</td>
<td>ATP syn 6</td>
<td>T8993G/C</td>
</tr>
<tr>
<td>Myoclonic Epilepsy Associated with Ragged-Red Fibers (MERRF)</td>
<td>Neurodegenerative / seizures / abnormal muscle development</td>
<td>LYS tRNA</td>
<td>A8344G, T8356C</td>
</tr>
<tr>
<td>Progressive External Ophthalmoplegia</td>
<td>Muscle weakness / droopy eyelids</td>
<td>LYS tRNA, ILE tRNA</td>
<td>A3243G, T4274C</td>
</tr>
<tr>
<td>Myopathy</td>
<td>LEU tRNA 2(CUN), GLU tRNA</td>
<td></td>
<td>T14709C, A12320G</td>
</tr>
<tr>
<td>Encephalomyopathy</td>
<td>VAL tRNA, GLY tRNA</td>
<td></td>
<td>G1606A, T10010C</td>
</tr>
<tr>
<td>Cardiomyopathy</td>
<td>LEU tRNA 1(UUA/G) ILE tRNA</td>
<td></td>
<td>A3243G, A4269G</td>
</tr>
<tr>
<td>Diabetes and deafness</td>
<td>LEU tRNA 1(UUA/G) SER tRNA 2(AGU/C)</td>
<td></td>
<td>A3243G, C12258A</td>
</tr>
<tr>
<td>Nonsyndromic sensorineural deafness</td>
<td>12S rRNA</td>
<td></td>
<td>A7445G</td>
</tr>
<tr>
<td>Aminoglycoside-induced nonsyndromic deafness</td>
<td>16S rRNA</td>
<td></td>
<td>A1555G</td>
</tr>
</tbody>
</table>

Information adapted from Chinnery, 2008

Figure 1A. Graphical representation of East Asian and Native American mitochondrial lineages

Information adapted from Álvarez-Iglesias, 2007
Author’s Biography:

Cassidy Punt began his pursuit of a Bachelor of Science Degree in Biochemistry from Minnesota State University, Mankato in the spring of 2002. His research began in the spring of 2005 with the help of Elizabeth Smalley, a Minnesota State University, Mankato alumna (2005) of the Department of Chemistry and Geology. In the summer of 2005, he was called to active duty with the Minnesota Army National Guard to serve in Iraq. Upon returning in the summer of 2007, he continued in his research and education within the Biochemistry program at Minnesota State University, Mankato. His research was completed in the spring of 2008 and was presented as a poster at the 10th Annual Undergraduate Research Conference at Minnesota State University, Mankato. Following graduation in the fall of 2008, Cassidy plans to earn a Ph.D. in human or medical genetics in order to pursue a career of his own in teaching and research.

Mentor’s Biography:

Dr. Terry Salerno received an A.B. in biochemistry from Mt. Holyoke College and a Ph.D in biochemistry from the University of Wisconsin, Madison. She has been a professor at MSU Mankato for over 20 years where she teaches allied health chemistry and biochemistry courses. In the past several years she has mentored several undergraduates in research. She is interested in curriculum development and likes to interface undergraduate research projects with laboratory development in the biochemistry curriculum. This research design was successfully applied to a biochemistry class project.