

# A High Incidence of Mitochondrial DNA Heteroplasmy in Hypervariable Region 1 in Normal Human Tissues: Implications for Forensic Casework

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## ABSTRACT

Mitochondrial DNA (mtDNA) sequence analysis of the hypervariable control region has been shown to be an effective tool for human identification. The high copy number and maternal mode of inheritance make mtDNA analysis particularly useful when advanced age or degradation of biological evidence prohibits the detection of nuclear DNA polymorphisms. Heteroplasmy, or the existence of subpopulations of mtDNA genomes within an individual, has been found to occur in the control region of normal humans. However, the frequency of heteroplasmy, as well as its impact on interpretation of forensic data, have not been established.

In order to better understand the occurrence and patterns of heteroplasmy in human tissues, we have implemented a denaturant gradient gel electrophoresis (DGGE) system designed to examine heteroplasmy in the hypervariable region 1 (HV1) portion of the mtDNA control region. DGGE separates DNA molecules based on their sequence, as opposed to their size, and our system was shown to be capable of detecting heteroplasmic variants at levels as low as 1%. We used this DGGE assay to screen for heteroplasmy in a total of 104 postmortem tissues, including bone, blood, hair, brain, liver, and muscle, from 21 normal adult individuals. When heteroplasmy was detected, the heteroplasmic position was identified by excising the DGGE bands, and eluting, re-amplifying, and sequencing the DNA. Additionally, the DNA from all heteroplasmic tissues was sequenced to examine the success of heteroplasmy detection by direct sequencing of PCR products.

Heteroplasmy in one or more tissues was observed in 11 individuals, or 52.4% (95% C.L. 31.0-73.8). The majority of heteroplasmic variants occurred at very low levels and were not detected by direct sequencing of original PCR products. Additionally, individuals with heteroplasmy at position 16093 demonstrated "flipped" heteroplasmic ratios, that is, the predominant species in one tissue was found to be the minor species in another.

This study demonstrated that HV1 heteroplasmy is a common occurrence in tissues from normal individuals, and should be considered in forensic cases where two samples appear to differ at a single nucleotide position by direct sequencing.

## SAMPLE POPULATION

Postmortem tissues, including bone, brain, liver, muscle, hair, and blood, were received from 21 individuals, ages 18-52. The racial distribution was 19 Caucasians and two African-Americans. All individuals had no known genetic disorders. Two-six tissues were analyzed from each individual, for a total of 104 tissues.

## SAMPLE ANALYSIS

Genomic DNA was isolated from whole blood using the Puregene DNA Isolation Kit (Gentra Systems, Inc., Minneapolis, MN), from hair samples using organic extraction, and from bloodstains and other tissues using Chelex 100 chelating resin (Biorad, Hercules, CA). Following extraction, all samples were stored at either 4°C or -20°C pending PCR amplification.

PCR amplification was performed using *Pfu* (*Pyrococcus furiosus*) polymerase (Stratagene, La Jolla, CA), because of its 3' – 5' exonuclease (proofreading) activity. HV1 was amplified as two overlapping fragments using primer sets F15989 (5' CCCAAAGCTAAGATTCTAAT 3')/ R16258GC (5' TGGCTTTGGAGTTGTAGTTG 3'), and F16144GC (5' GAGGATGGTGGTCAAGGGAC 3')/ R16410 (5' TGACCACCTGTAGTACATAA 3'). A GC clamp (GC) is a 40 base GC-rich sequence added to the 5' end of one primer in each set that is incorporated into the resulting PCR product, making it suitable for DGGE analysis. Approximately 10-50 ng of DNA template were added to each PCR reaction, along with 1X *Pfu* reaction buffer (20mM Tris-HCl pH 8.8, 2mM MgSO<sub>4</sub>, 10mM KCl, 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.1% Triton X-100, 0.1 mg/ml nuclease-free BSA), 200 μM each dNTP (Boehringer Mannheim Corp., Indianapolis, IN), 0.2 μM each primer (Synthetic Genetics, San Diego, CA), 2 μg bovine serum albumin (8

µg for hair samples) (Sigma Chemical, St. Louis, MO), 2.5 Units of *Pfu* Polymerase (5 Units for hair samples), and sufficient sterile distilled water (Gibco BRL Life Technologies, Gaithersburg, MD) to bring the final volume to 50 µl. PCR amplification was performed using an initial denaturation of 95°C for 30 seconds, followed by 35 cycles of 95°C for 30 seconds (denaturation), 50°C (F15989/R16258GC) or 60°C (F16144GC/R16410) for 30 seconds (primer annealing), and 72°C for 30 seconds (extension).

Denaturant gradient gel electrophoresis (DGGE) was performed using a 6.5% polyacrylamide gel with a 40-50% chemical denaturant (urea/ formamide) gradient. Electrophoresis was performed at 100 volts at 60°C for 15 hours. Upon completion, the gels were stained with SYBR Green (Molecular Probes, Eugene, OR) for 20 minutes. Visualization of the DNA bands was performed using an ultraviolet wavelength transilluminator. The gels were then photographed using Polaroid 667 black and white film.

## HETEROPLASMY DETECTION AND CHARACTERIZATION

Heteroplasmy detection by DGGE has been described elsewhere (1,2). Briefly, prior to analysis by DGGE, the PCR product is heated to 95°C to denature the DNA, then slowly cooled to room temperature to allow reannealing of the DNA strands. When all DNA sequences are identical (homoplasmy), a single *homoduplex* (two strands perfectly base-paired) will form, resulting in a single band on a DGGE gel. When DNA sequences differ by a single base pair (heteroplasmy), four types of DNA duplexes will form: two *homoduplexes*, representing the heteroplasmic species, and two hybrid *heteroduplexes* which are comprised of a strand from each original DNA duplex. The heteroduplexes contain a base-pair mismatch at the heteroplasmic site and are easily resolved from the homoduplexes due to differences in mobility in the denaturant gradient. Validation studies have demonstrated that this DGGE assay was capable of detecting heteroplasmic proportions as low as .05 (1/20) to .01 (1/100), depending on the position.

Tissue samples which were homoplasmic for HV1 sequences were not further analyzed. Samples appearing to be heteroplasmic were initially re-analyzed to confirm the results. The heteroplasmic positions were then identified by excising the homoduplex bands from the DGGE gel, and eluting, re-amplifying, and sequencing the DNA using the ABI PRISM® Dye Terminator Cycle Sequencing Kit Taq FS, and the ABI 310 Capillary Electrophoresis Analyzer (Perkin Elmer, Foster City, CA). When the proportion of the minor heteroplasmic species was too low to be visualized in the homoduplex form, one or both heteroduplexes were excised from the

DGGE gel, re-amplified (re-amplification of heteroduplex DNA yields a product containing approximately equal proportions of both heteroplasmic species), and analyzed by a second round of DGGE. Second round DGGE resulted in separation and visualization of both homoduplex bands, due to enrichment of the minor species during the re-amplification process. The resulting bands were excised, re-amplified, and sequenced as described above, allowing identification of the heteroplasmic sequence.

To estimate the heteroplasmic proportions, the negative image of the Polaroid photograph of the DGGE gel was scanned using the Hewlett Packard 3J scanner, and analyzed using NIH Image software. The relative proportions of each heteroplasmic species were determined based on the density of each band. Finally, the original PCR product from each heteroplasmic tissue sample was sequenced to determine the success of heteroplasmy detection by direct sequencing of PCR products.

## RESULTS AND DISCUSSION

Heteroplasmy in one or more tissues was detected in 11 individuals, or 52.4% (95% C.L. 31.0 – 73.8). The sensitivity of DGGE, as well as the availability of multiple tissues from each individual, allowed a more comprehensive investigation of HV1 heteroplasmy than was possible by previous studies which examined blood samples by direct sequencing of PCR products (3) and by single strand conformational polymorphism analysis (4). As a result, our study found a high incidence of HV1 heteroplasmy in normal human tissues. Furthermore, the majority of these heteroplasmic variants could not be detected by sequencing. Although a relatively small number of individuals (n = 21) were examined, these results clearly demonstrate that, up until now, the frequency of heteroplasmy in the mtDNA control region has been underestimated.

Several patterns of heteroplasmy were observed in individuals in this study. These included multiple heteroplasmic positions, heteroplasmy in just a subset of tissues, and substantial differences in heteroplasmic proportions among tissues. This last finding was specific to three individuals with heteroplasmy at position 16093. For example, one individual was found to be heteroplasmic at position 16093 (C/T) in the five tissues (blood, bone, hair, brain, muscle) analyzed. The proportions of cytosine and thymine varied among these tissues, and as demonstrated in the DGGE gel (Figure 1), the predominant sequence in bone and muscle was the minor sequence in blood and hair. Inspection of the sequencing data from this individual (Figure 2), however, demonstrates that direct sequencing of PCR products failed to detect heteroplasmy in muscle, hair,

and blood, and in fact, identified muscle as homoplasmic for thymine and hair and blood as homoplasmic for cytosine. These “flipped” heteroplasmic ratios were not observed at any other position. However, a small number of individuals were studied, and additionally, this study was limited to HV1. It is not known whether any positions in hypervariable region 2 (HV2) exhibit this same phenomenon.

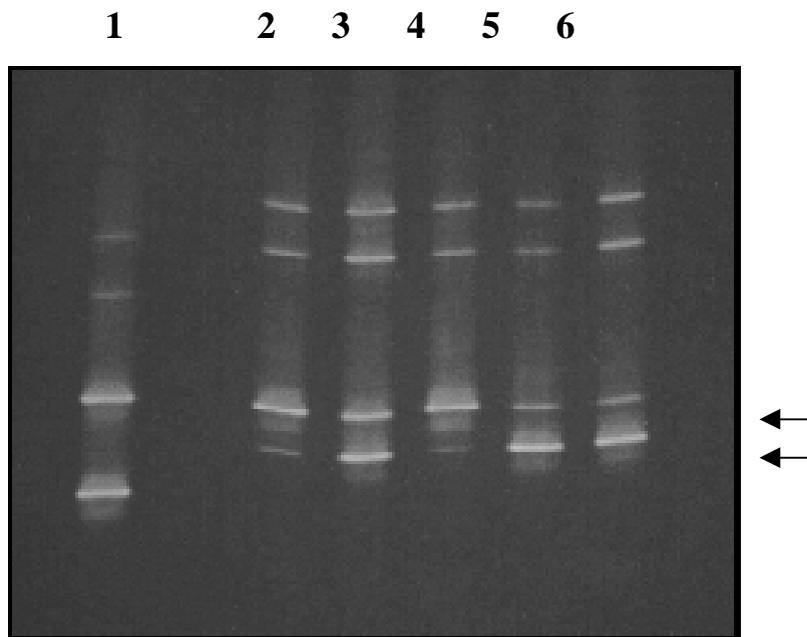
This study demonstrates that the existence of heteroplasmy should be considered in forensic cases where two samples appear to differ at a single nucleotide position by direct sequencing. The use of DGGE may help resolve these cases, allowing a definitive conclusion to be drawn.

The authors gratefully acknowledge the University of Maryland Brain and Tissue Bank (NIH contract number NO1-HD-1-3138) for providing the tissue samples used in this study.

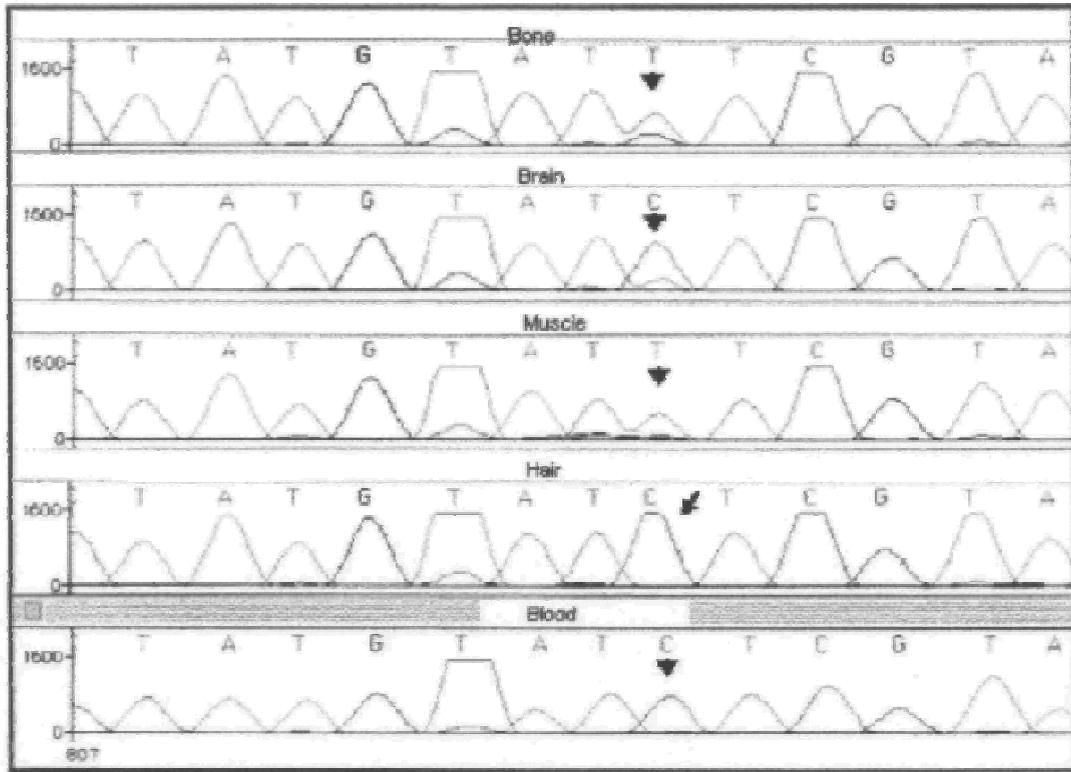
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**Figure 1:** DGGE gel from an individual with 16093 C/T heteroplasmy in five different tissues. The two homoduplex bands in each lane (designated by arrows) represent the heteroplasmic species, which were subsequently identified by excising the bands, and eluting, re-amplifying, and sequencing the DNA. These results demonstrate that in bone and muscle, thymine was the predominant sequence (upper band more intense), whereas in hair and blood, cytosine was the predominant sequence (lower band more intense). (Lane 1, heteroplasmic control; Lane 2, bone; Lane 3, brain; Lane 4, muscle; Lane 5, hair; Lane 6, blood).



**Figure 2:** Direct sequencing of the PCR products from the tissues analyzed by DGGE in Figure 1. Bone and brain appear to be heteroplasmic at position 16093 (designated by arrows) by direct sequencing. However, muscle appears to be homoplasmic for thymine, whereas hair and blood appear to be homoplasmic for cytosine.