JOURNAL OF FORENSIC SCIENCES

J Forensic Sci, May 2010, Vol. 55, No. 3
doi: 10.1111/j.1556-4029.2010.01339.x
Available online at: interscience.wiley.com

TECHNICAL NOTE

CRIMINALISTICS

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Heteroplasmy in Hair: Study of Mitochondrial DNA Third Hypervariable Region in Hair and Blood Samples*†

ABSTRACT: Mitochondrial DNA (mtDNA) analysis has proved useful for forensic identification especially in cases where nuclear DNA is not available, such as with hair evidence. Heteroplasmy, the presence of more than one type of mtDNA in one individual, is a common situation often reported in the first and second mtDNA hypervariable regions (HV1/HV2), particularly in hair samples. However, there is no data about heteroplasmy frequency in the third mtDNA hypervariable region (HV3). To investigate possible heteroplasmy hotspots, HV3 from hair and blood samples of 100 individuals were sequenced and compared. No point heteroplasmy was observed, but length heteroplasmy was, both in C-stretch and CA repeat. To observe which CA "alleles" were present in each tissue, PCR products were cloned and re-sequenced. However, no variation among CA alleles was observed. Regarding forensic practice, we conclude that point heteroplasmy in HV3 is not as frequent as in the HV1/HV2.

KEYWORDS: forensic science, mitochondrial DNA, heteroplasmy, hair, blood, HV3

Heteroplasmy, the presence of more than one type of mitochondrial DNA (mtDNA) within an individual, is a situation found more commonly in hair than in blood samples (1–4). The clonal nature of hair follicles and the high-energy requirements of keratinizing hair shaft cells are two features of hair histogenesis that could contribute to the high observation of the segregation of heteroplasmic variants in mtDNA from hair shafts (5). In contrast, peripheral-blood samples consist of lymphocytes produced by a very large number of hemopoietic stem cells in the bone marrow, and if proportions differ in individual stem cells, the heteroplasmy measured is an averaged value of the proportions of all stem cells. The high mutation rate of the mtDNA, when compared to nuclear DNA, appears to occur because it is more exposed to mutagenic events, owing to its lack of protective histones, proximity to the respiratory chain, and its relative deficiency in DNA repair mechanisms (6).

Heteroplasmy can be differentiated into two types: point and length heteroplasmy. A sequence point, or site heteroplasmy, is defined as sequences containing different bases at the same position. The sequence electropherogram usually shows two different bases superimposed (7). A length heteroplasmy is represented by multiple populations of mtDNA containing repetitive stretches (typically C-stretches or CA repeats) of various lengths.

The presence of heteroplasmy in a forensic case can be relevant because two samples from the same individual may differ in levels of heteroplasmy or sometimes in a homoplasmic substitution (8). Therefore, it is important to determine the frequency of heteroplasmy and hotspots, to avoid incorrect interpretations.

While a number of studies have been published on heteroplasmy in the first and second hypervariable regions (HV1 and HV2), little is known about the third hypervariable region (HV3). Its importance to forensic science was described by Lutz et al. (9), and it is now incorporated into forensic analysis, mainly because of an informative dinucleotide repeat (CA)n (10–15). Analysis of the entire mitochondrial DNA control region, including HV3, is also being proposed, for the best determination of the haplogroup affiliation of populations. Phylogenetic analysis is being used for quality assurance of sequences (16). At this time, there are no data about possible hotspots of heteroplasmic positions and their frequencies in the HV3 region, comparing hair and blood samples.

The aim of our work was to find differences in the HV3 (nt 438-574) sequences between hair and blood from the same

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*Presented at the DNA in Forensics 2008 Conference, May 27-30, 2008, in Ancona, Italy.

†Funded by a grant from FAPESP - Fundação de Apoio a Pesquisa do Estado de São Paulo (2005/03984-5). G.G. Paneto receives a fellowship from CNPq – Conselho Nacional de Pesquisa (2007-0/140052), and L.V.G. Longo received a fellowship from FAPESP (2006/06772-1). CNPq is the Brazilian National Research Council and FAPESP is the São Paulo State Research Aid Foundation.

Received 11 May 2008; and in revised form 1 May 2009; accepted 3 May 2009.

individuals to determine possible hotspots of heteroplasmic positions and the frequencies in those tissues.

Materials and Methods

DNA Samples and Extraction

One hundred unrelated Brazilian individuals were analyzed (all the participants gave their written informed consent prior to their inclusion in this study). Samples from head hair and blood (spotted onto filter paper and air-dried) were collected from the same individuals (3–6 years previously), and stored at 8°C (in refrigerator) until analyzed.

Hair shafts were cut off 0.5 cm above the skin surface, and 4 cm of the hair was cut into pieces of 0.5 cm each (more than one hair was used when the length of hair was smaller than 4 cm). Hair samples were decontaminated as described by Jehaes et al. (17) prior to DNA extraction. DNA was extracted using Tissue and Hair extraction kit (Promega, Madison, WI), with the following modifications: incubation for 2 h 30 min, followed by phenyl/chloroform/isoamyl alcohol (PCIA) extraction (USB-GE GE, Chalfont St. Giles, UK) and purification with columns of illustra GFX™ PCR DNA and gel band purification kit (GE). DNA was eluted in 50 μL double-distilled H_2O (ddH_2O).

DNA was extracted from blood with the DNA IQ™ System kit (Promega), following the manufacturer's instructions to obtain a final concentration of 2 ng DNA/μL.

PCR Amplification

Amplification was carried out on 10 μL of hair extraction product or 4 ng of blood DNA template in a 50-μL reaction containing each of the following primers at 0.2 mM: HV3-F314 (5′-CCG CTT CTG GCC ACA GCA CT-3′) and HV3-R639 (5′-GGG TGA TGT GAG CCC GTC TA-3′) (IDT, Coralville, IA), 5 μL of GoldSTAR® Buffer (Promega), and 0.4 μL of AmpliTaqGold® DNA polymerase (Applied Biosystems, Foster City, CA), made up to the volume with ddH_2O. Thermal cycling was performed in a GeneAmp® PCR System 9700 (Perkin-Elmer, Wellesley, MA) starting at 95°C, for 11 min, then 1 min at 96°C, followed by 40 cycles at 94°C for 30 sec, 30 sec at 56°C, and 90 sec at 72°C, ending with 11 min at 60°C. Amplicon yield was evaluated by running 5 μL of all the PCR product reaction and comparing it with 2 μL of Low DNA Mass™ Ladder (Invitrogen, Carlsbad, CA) on 1% agarose gel, stained with ethidium bromide, to estimate the PCR product concentration. Negative controls were included in the extraction and PCR reactions, to detect any possible contamination. PCR products were cleaned and concentrated (to at least 20 ng/μL) with illustra GFX™ PCR DNA and gel band purification kit (GE).

Sequencing

Cycle sequencing of both strands was carried out using BigDye® Terminator Cycle Sequencing Ready Reaction Kit v1.0 (Applied Biosystems), using the same primers as in the PCR reaction. Reaction products were ethanol precipitated and electrophoresed in an ABI PRISM® 377 Genetic Analyzer (Applied Biosystems).

Data Analysis

Data analysis was carried out with BioEdit software (18). Only high quality sequences with little or no background signal were used. Sequences were aligned with the Revised Cambridge Reference Sequence. Point heteroplasmy was only accepted if a secondary peak of more than about 10% of the primary peak was present and confirmed in the reverse sequencing reaction. The proportion of the secondary peak was estimated by measuring the heights of the primary and secondary peaks of mixed basecalls, as depicted in the electropherograms, with at least two independent PCR reactions, to rule out sequencing artifacts or mixtures.

Cloning

PCR products containing CA heteroplasmy (electropherograms "out of phase") were cloned into the pGEM®-T Vector (Promega), to isolate each allele. Plasmid DNA was isolated by using the protocol described by Sambrook et al. (19).

Results and Discussion

Point Heteroplasmy

No difference among hair and blood from the same individuals was observed by direct PCR product sequencing and no point heteroplasmy was observed. Irwin et al. (20) and Zimmermann et al. (21) reported point heteroplasmy at positions 448R and 498Y, respectively, in blood samples. By contrast, no data has been published to date regarding heteroplasmy in the HV3 region in hair samples. Heteroplasmy had been shown to be frequent in hair samples in the HV1 and HV2 regions (3,22) and we expected that it would also occur, to some extent, in HV3. In general, the sites at which heteroplasmy is most commonly observed are correlated with reported control region mutational hotspots. According to a population study of 200 unrelated individuals from Germany, HV1 (np 16,024–16,365) showed 88 variable positions in a total length of 342 bp (26%) and HV2 (np 73–340) displayed 65 mutable sites in 268 bp (24%). HV3 (np 438–574) exhibited a slightly lower variability, with 25 polymorphic sites within 137 bp (18%), but contrasted clearly with the intervening regions, which, respectively, showed variability rates of only 7% (np 16,366–16,569, 1–72) and 3% (np 341–437) (9). In a Japanese study, in HV1, HV2, and HV3, 80, 37, and 14 polymorphic sites were identified, respectively, representing 23, 14, and 10% of the total size of each region, excluding all C-stretches (23). Our population showed 22 polymorphic positions in HV3 (data not shown), excluding all C-stretches, representing 16% of the total size of the region. Apparently, because HV3 is a less polymorphic region than HV1/HV2, it appears to be less prone to mutation (9,24). At some sites, however, the observation of heteroplasmy is not consistent with established mutation rate data, suggesting the action of other mechanisms, both selective and neutral, as occur at position 16,093. However, we did not find any vestige of point heteroplasmy in our samples.

Length Heteroplasmy

Concerning length heteroplasmy, however, one individual showed heteroplasmy in the CA repeat (in both blood and hair samples) with "out of phase" electropherogram (Fig. 1). This result, regarding blood samples, is consistent with earlier studies. Szibor et al. (13) found 1.38% of CA heteroplasmy in a German population and Chung et al. (25) found 0.6% in a Korean population. No information about its frequency in hair samples has been published to date. As for the mechanism of heteroplasmy formation in the CA dinucleotide repeats, their propensity for insertion/deletion mutation of multiples of the repeating unit during replication

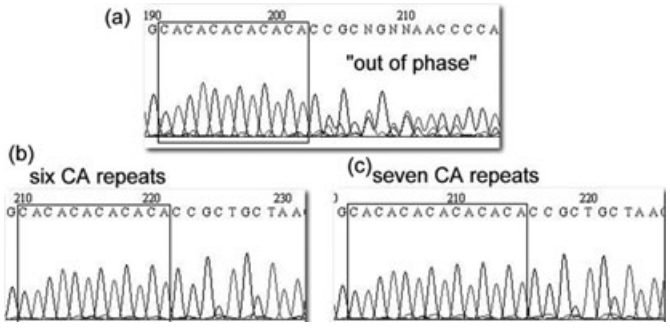

FIG. 1—*(a) CA heteroplasmy found in hair and blood of the same individual with electropherogram "out of phase" after CA repeat. (b–c) Two different alleles that compound CA heteroplasmy separated after cloning.*

could explain the phenomenon, as could the low fidelity of mitochondrial DNA polymerase. A high slippage rate of dinucleotide repeats can be observed in STRs, and the resultant stutter production is correlated with the length of repeat stretches consisting of uniform repeats (26). Length heteroplasmy was also observed at position 573 (poly-C stretch) in three individuals.

To observe which CA "alleles" were present in each tissue containing CA heteroplasmy, PCR products were cloned and re-sequenced. The presence of mixed alleles would suggest the possibility of an intra-individual difference in CA repeats between hair and blood samples. However, no variation among CA alleles was observed in hair and blood, even after cloning and re-sequencing. Recently, some authors reported unstable inheritance of HV3 heteroplasmy variants over a few generations. Szibor et al. (13) showed unstable inheritance at the CA repeat locus when analyzing two pedigrees. Brandstätter et al. (27) reported one case of CA divergence in 135 families (mother–child pairs). In general, this could be because of a marked reduction in the number of mtDNA molecules being transmitted from mother to offspring (the mitochondrial genetic bottleneck), to the partitioning of mtDNA into homoplasmic segregating units, or to the selection of a group of mtDNA molecules to populate the next generation. Cree et al. (28) recently suggested that the partitioning of mtDNA molecules into different cells before and after embryo implantation, followed by the segregation of replicating mtDNA between proliferating primordial germ cells, is responsible for the varying levels of heteroplasmy seen among the offspring of heteroplasmic females, at least in mice.

Conclusion

Regarding forensic practice, we have seen that point heteroplasmy in HV3 is not as frequent as in the HV1 and HV2 regions. The contribution of HV3 analysis, however, could play a role in a forensic case. This region can increase discrimination and help in phylogenetic analysis for quality assurance of sequences. Also, the great genetic diversity in the CA repeat seems to increase its potential contribution to the forensic analysis. Nevertheless, because there have been few studies concerning variations of CA among different tissues within the same individual, its analysis should be used with caution.

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