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Research Paper

Assessing heteroplasmic variant drift in the mtDNA control region of human hairs using an MPS approach

Jamie M. Gallimore, Jennifer A. McElhoe, Mitchell M. Holland*

Forensic Science Program, Department of Biochemistry and Molecular Biology, The Pennsylvania State University, 014 Thomas Building, University Park, PA 16802, United States

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ABSTRACT

Resolution of mitochondrial (mt) DNA heteroplasmy is possible when applying a massively parallel sequencing (MPS) approach. However, interpretation criteria for matching heteroplasmic sequences will need to be established that address a number of important topics, including the drift of variants in sample types such as human hair shafts. Prior to MPS analysis, we compared three different DNA extraction methods for hair using a custom mtDNA quantitative PCR (mtqPCR) assay, and found that a method involving bead capture significantly outperformed methods currently in place in forensic laboratories. The findings were similar for both fine (head) and coarse (pubic) hairs. Using the favored DNA extraction approach, hair shaft extracts were subjected to MPS analysis to assess heteroplasmic drift and the potential impact of the observations on interpretation of mtDNA MPS data. Hairs from different regions of the head were evaluated in individuals with varying percentages of heteroplasmy (low-level, high-level, and no detectable heteroplasmy), as measured in buccal and blood cells. The range of variant ratios was broad and was not significantly different between individuals in the low and high-level groups. While the range was also broad for the group of individuals with no heteroplasmy, the vast majority of hairs from these donors still exhibited a lack of heteroplasmy. A model was developed to predict the amount of heteroplasmy expected in hair samples when knowledge of the percentage of heteroplasmy in buccal cells is available. While significant, the model was best applied when levels of heteroplasmy in buccal cells was high. No correlation was observed between rates of heteroplasmy in blood cells and the predicted amount of heteroplasmy in hairs. Of particular interest, unexpected sites of mixed mtDNA sequence that could be interpreted as heteroplasmy were observed for 13% of the 75 hairs tested. These sites can be explained as heteroplasmy not observed in buccal or blood cells, or sites of DNA damage, with inherent heteroplasmy a likely cause, possibly due to de novo mutation events. Overall, when applying an MPS approach to hair analysis, heteroplasmic variant ratios may be quite different than those observed in blood cells, may be correlated to rates in buccal cells, and may include unexpected mixed sites. The results of this study directly impact MPS analysis of minor sequence variants from hair samples, and are particularly relevant to clinical and forensic investigations.

1. Introduction

Hair is a common evidence type found at crime scenes, and is an emerging sample type for medical research investigations [1,2]. Both anagen and catogen hairs often have associated follicular tissue, especially when forcibly removed from the head or body, leading forensic laboratories to conduct nuclear (n) DNA analysis on root tissue. Telogen hairs, on the other hand, are readily shed from the body and lack follicular tissue. As a result, mitochondrial (mt) DNA sequencing is used to replace nDNA testing due to the limited nDNA content found in a telogen hair root, or in the shaft of a hair during any growth phase. The analysis of mtDNA sequence in forensic investigations has been a routine practice for more than twenty years [3].

Forensic analysis of hair shafts begins with DNA extraction, arguably the most important step in the process. Studies involving the development of extraction methods have claimed to provide greater DNA yields than other commonly used methods [4–7]. To the best of our knowledge, a comparison study of extraction protocols used by forensic laboratories has not been published. Following DNA extraction and amplification of target segments of the control region (CR), sequencing of the amplicons has traditionally been performed with Sanger type sequencing (STS) [3]. The outcome is an mtDNA haplotype and the possible detection of heteroplasmy, the presence of additional sequence variants within an individual. One in every 15 forensic cases may

* Corresponding author.
E-mail address: mmh20@psu.edu (M.M. Holland).

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include the observation of heteroplasmy when using an STS approach [8], as heteroplasmy is observed quite often in hairs [1,9–11]. However, while STS is able to detect heteroplasmy at a threshold of 10–20%, it is unable to resolve the variants without further analysis; for example, cloning or denaturing gradient gel electrophoresis [3]. In contrast, massively parallel sequencing (MPS) is able to both detect and resolve heteroplasmy at levels as low as 1–2% [12–14].

If resolved, the observation of heteroplasmy will increase the discrimination potential of a resulting match between items of evidence and a reference source. A notable example is the identification of Nicholas Romanov II, the last Russian Tsar [15,16]. A heteroplasmic position observed in the remains of the Tsar was also observed in the mtDNA CR of his brother, Georgij Romanov. The likelihood ratio of the haplotype alone was 150, but increased to 380,000 when the heteroplasmic event was included in the calculations, illustrating the value of assessing heteroplasmy in forensic investigations. Ongoing studies to measure the rates of heteroplasmy at the nucleotide and individual levels in different major population groups, providing the basis for weight estimates, will ultimately allow for this application [17]. For example, the rate of heteroplasmy in a European population group has been measured empirically [18], suggesting that one in every two or three forensic cases will include the interpretation of heteroplasmy when using an MPS approach.

The high copy nature of the mtgenome translates into a situation where mtDNA mutations pass through a state of heteroplasmy until fixation, or reversion back to the original nucleotide. Only mutations originating in germ cells will be transmitted to subsequent generations through the female germline. The narrow bottleneck of germline transmission allows for increased drift in the ratio of heteroplasmic variants between maternal relatives [19,20]. This holds true for tissue types within an individual [9–11,21], driven by the cellular differentiation process of a developing fetus, and the random distribution of heteroplasmic variants. A notable example of this is a recently reported mini-pig model [22]. This study evaluated four heteroplasmic positions across three generations originating from the same maternal ancestor. In agreement with other studies, the pig model showed significant differences in the ratio of variants at one heteroplasmic position across three generations in six different tissue types: brain, diaphragm, muscle, liver, heart, and adipose tissue. While these studies provide information regarding the drift of heteroplasmy in different tissue types, there have been relatively few studies of heteroplasmy or heteroplasmic drift in human hairs [for example,1,9–11,23], and most have involved STS.

The current study evaluated mtDNA heteroplasmy in the CR in three tissue types; blood and buccal cells, and hair shafts. During early histogenesis, three germ layers (ectoderm, mesoderm, and endoderm) differentiate from one another. Blood originates from the mesoderm layer while hair and buccal cells originate from the ectoderm layer. Given that buccal cells and hair are more closely related, we hypothesized that rates of heteroplasmy in hair shafts could be predicted by assessing rates in buccal cells. However, this relationship could be complicated by the nature of variant drift between the follicles of an individual, as each event of establishing the cells in the hair peg involves a bottleneck [24]. Unlike blood or buccal cells, individual hairs originate from follicles or clusters of cells that have independent growth cycles. Each follicle is formed from a cluster of cells in the ectoderm at 9–12 weeks post gestation, providing an opportunity for additional layers of variant drift. This effect has not only been observed between hairs, but also along the length of the hair shaft using STS [11,14,25], prompting some to suggest that the high level of drift complicates the interpretation of matching haplotype information [17]. Therefore, an evaluation of drift in human hair shafts using an MPS approach is needed to allow for the reporting of heteroplasmy in forensic cases.

The current study evaluated three DNA extraction protocols for human head and pubic hair shafts using a custom mtDNA quantitative PCR (mtqPCR) assay to identify the best method. The preferred approach was used to extract DNA from head hairs of individuals with varying levels of heteroplasmy to determine the potential impact of variant drift on the interpretation of MPS data, including the potential correlations between the rates observed in blood and buccal cells when compared to hair samples. To our knowledge, this is the first study of inter and intra-individual drift of heteroplasmic variants in human hair shafts using an MPS approach.

2. Materials and methods

2.1. Sample collection and selection

All hair, blood, and buccal samples were collected under the Penn State University internal review board (IRB) approved protocol STUDY00000970.

For the comparison study of DNA extraction methods, head and pubic hairs were collected from two different sets of five donors and stored at room temperature. All head hairs were brown in color and the donors were of European ancestry. Cuttings of 0.5 and 2.0 cm were taken from individual hairs from each donor. Each hair was sampled approximately 5.0 cm away from the root end. For the pubic hair evaluation, all hairs were curly and dark brown to black in color, and all donors were of European ancestry. Cuttings of 0.5 cm were taken from individual hairs from each donor. Each hair was cut approximately 1.0 cm away from the root end.

Given our measured rate of observing heteroplasmy in the CR using an MPS approach, for the drift study we screened blood and buccal samples from 38 individuals to identify five with no heteroplasmy ($>$ 1%), five with low-level reportable heteroplasmy (2–10%), and five with high-level heteroplasmy ($>$ 10%). All 76 samples were organically extracted; lysis with a stain extraction buffer [2% SDS (Amresco; Solon OH), 10 mM EDTA (Promega; Madison, WI), 100 mM NaCl (Dot Scientific; Burton, MI), 7.6 mM Tris-HCl (Quality Biological; Gaithersburg, MD, pH 8.0)] and proteinase K (20 mg/mL; Life Technologies, Carlsbad, CA), phenol-chloroform-isoamyl alcohol (PCIA)(ThermoFisher Scientific, Carlsbad, CA) purification, resuspension following ethanol precipitation in 50 uL of low TE (1 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) (Sigma-Aldrich, St. Louis, MO), and storage of extracts at −20 °C. Five donors were identified without heteroplasmy in blood and buccal cells; 1% threshold for detection, 2% for reporting. In the low-level group, four individuals had 2–10% heteroplasmy in both buccal and blood cells at one site, while a fifth donor had more than 10% heteroplasmy in their buccal cells and less than 10% in their blood. The average between the two samples was less than 10% which placed the fifth donor in the low-level group. In the high-level group, three of the five donors had greater than 10% heteroplasmy at one site in their blood and buccal cells. The last two donors had one sample (blood or buccal) with 2–10% heteroplasmy, while the remaining sample had greater than 10% heteroplasmy. The respective averages of the two sample types was above 10%, placing these last two donors in the high-level group. Table 1 lists the nucleotide position of the heteroplasmic single nucleotide polymorphism (SNP) in the mtDNA CR for each donor (shaded cells) along with the percentage of the minor variant; see Supplemental Table 1 for the haplotypes of each donor. As illustrated in Table 1, a subset of the donors exhibited more than one heteroplasmic SNP site. The non-shaded cells in the table are associated with these secondary heteroplasmic variants. Five head hairs, one each from the forehead, crown, neck, left temple, and right temple were collected from each of the 15 donors identified for the drift study. A 2.0 cm cutting was taken from each hair approximately 1 cm from the root end and extracted for MPS analysis with the LMB DNA extraction method described below.

2.2. Comparison of DNA extraction methods for hair shafts

The main elements of each DNA extraction method are summarized

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