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Q1 # Evidence for frequent and tissue-specific sequence heteroplasmy in human mitochondrial DNA

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ABSTRACT

Mitochondrial point heteroplasmy is a common event observed not only in patients with mitochondrial diseases but also in healthy individuals. We here report a comprehensive investigation of heteroplasmy occurrence in human including the whole mitochondrial control region from nine different tissue types of 100 individuals. Sanger sequencing was used as a standard method and results were supported by cloning, minisequencing, and massively parallel sequencing. Only 12% of all individuals showed no heteroplasmy, whereas 88% showed at least one heteroplasmic position within the investigated tissues. In 66% of individuals up to 8 positions were affected. The highest relative number of heteroplasmies was detected in muscle and liver (79%, 69%), followed by brain, hair, and heart (36.7%–30.2%). Lower percentages were observed in bone, blood, lung, and buccal cells (19.8%–16.2%). Accumulation of position-specific heteroplasmies was found in muscle (positions 64, 72, 73, 189, and 408), liver (position 72) and brain (partial deletion at position 71). Deeper analysis of these specific positions in muscle revealed a non-random appearance and position-specific dependency on age. MtDNA heteroplasmy frequency and its potential functional importance have been underestimated in the past and its occurrence is ubiquitous and dependent at least on age, tissue, and position-specific mutation rates.

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1. Introduction

Human mitochondrial DNA (mtDNA) is inherited maternally (Birky, 1995, 2001; Hutchison et al., 1974). Owing to this uniparental and non-recombinant mode of inheritance, theoretically only a single mtDNA sequence (mtDNA haplotype) is present in an individual and in members of a maternal lineage. However, mtDNA is abundant and highly mutable, with an estimated 6–17-fold higher mutation rate than nuclear DNA (Brown et al., 1979, 1982; Miyata et al., 1982; Pesole et al., 1999; Wallace et al., 1987; Yakes and Van Houten, 1997). Thus, new mitochondrial haplotypes occur by point mutations as well as deletions or insertions. As a consequence, many individuals can carry more than one haplotype, leading to a mixture of variant mtDNA genomes, termed heteroplasmy (Alonso et al., 2002; Melton, 2004). In the present study, we investigated the occurrence of point heteroplasmy, which is manifested as mixed nucleotide calls at single positions. Point heteroplasmy differs from length heteroplasmy that is evident from length differences, typically found in homopolymeric nucleotide tracts (Bandelt and Parson, 2008). Within the paper the word "heteroplasmy" refers to point heteroplasmy if not stated otherwise.

Heteroplasmy was originally believed to be associated with pathological states and rare in healthy individuals (Monnat and Loeb, 1985; Monnat and Reay, 1986; Tully et al., 2000). However, in recent years, Q3 studies have indicated that heteroplasmy also occurs in asymptomatic humans (Tully et al., 2000). Next-generation sequencing technologies (massively parallel sequencing, MPS) confirmed the hypothesis of a widespread appearance of heteroplasmy in healthy humans (Payne et al., 2013). Moreover, reports of an increase in somatic mtDNA mutations with aging and the incidence of tissue-specific mutations have been published (Calloway et al., 2000; Goto et al., 2011; Jazin et al., 1996; Lacan et al., 2009; Lee et al., 2006; Michikawa et al., 1999; Samuels et al., 2013; Wang et al, 2001).

Heteroplasmy appearing within the maternal oocyte, for instance resulting from a germ line mutation, may be transferred to the next generation. It might be expected that the mitochondrial haplotypes would become more and more heterogeneous over generations and

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that this variation would be detectable within all tissues. However, individuals showing multiple (visible) heteroplasmies in buccal cells or blood by Sanger sequencing are the exception rather than the rule. One possible explanation for this phenomenon is the existence of a bottleneck during some stages of oogenesis (Carling et al., 2011). In this scenario, only a small group of mtDNA molecules repopulates the next generation, leading to loss or rapid fixation of a new mutation. Several cases of a rapid switch from a heteroplasmic state to an apparent homoplasmic haplotype between (grand-) mother and offspring have been reported in humans (do Rosario Marinho et al., 2011; Goto et al., 2011; Lo et al., 2005; Sekiguchi et al., 2003). As a result, mtDNA shows a high level of diversity between maternally unrelated individuals and therefore, many different mtDNA haplogroups have evolved over time.

Heteroplasmy does not occur randomly within the mitochondrial control region, but is concentrated in certain positions called mutational hotspots (Bandelt et al., 2002; Excoffier and Yang, 1999; Hasegawa et al., 1993; Irwin et al., 2009; Jazin et al., 1998; Malyarchuk et al., 2002; Meyer et al., 1999; Salas et al., 2005; Stoneking, 2000; Wakeley, 1993). Some mutations are less stable and have back-mutated over short evolutionary time (Duggan and Stoneking, 2013). Additionally, positive and negative selection of new mutations could alter mitochondrial functions, with different effects dependent on the tissue. Tissue-specific mutations in MPS have recently been seen in a few individuals (Samuels et al., 2013). Possible correlations of heteroplasmy with location, inheritance, age, population group, tissue type, gender, or disease are still controversial and under investigation (Avital et al., 2012; Coskun et al., 2004; de Camargo et al., 2011; do Rosario Marinho et al., 2011; He et al., 2010; Lagerström-Fermér et al., 2001; Rose et al., 2010; Wang et al., 2001).

In this study, we investigated the occurrence of heteroplasmy within the whole non-coding control region (CR) using nine different tissues from 100 randomly selected individuals, with the following aims. First, we determined the frequency of heteroplasmy by Sanger sequencing and confirmed questionable cases or, in case of unusual accumulations of heteroplasmic positions, by further techniques including cloning, minisequencing, and MPS. Second, we investigated if and to what extent tissue-specific differences occur, and if so, which positions are concerned. Third, we investigated if heteroplasmy in general or selected heteroplasmic positions are dependent on age, gender, haplogroup, or body mass index (BMI).

2. Methods

2.1. Samples and DNA extraction

Buccal swabs and samples of blood, hair, bone, skeletal muscle, heart muscle, brain, lung, and liver from 100 deceased individuals without signs of decay were taken during routine autopsy. Approval was provided by the ethics committee of the University of Freiburg (statement 264/10, August 26, 2010). Information on age, weight, gender, cause of death, and noted indications of diseases were collected. DNA was extracted from all samples using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocols for tissues and hair. For bones, a modified protocol was applied. Prior to extraction, samples were prepared as follows: half of the buccal swabs were placed in reaction tubes and blood samples of 200 μL were transferred in a tube. Soft tissues were washed over night with PBS (Life Technologies (LT), Carlsbad, CA, USA). The following day, small pieces (approximately 8 mm^3) were excised from the inner side of the tissue. Five hairs with roots (if available) were assembled and washed with ATL buffer (Qiagen, Hilden, Germany), EtOH (80%) and HPLC water. Bones were separated from soft tissues and washed overnight with washing powder (Persil, Henkel, Düsseldorf, Germany) and soda at 50 °C. They were cut into small pieces and cleaned with HPLC water, EtOH (80%), and UV light.

At the Institute of Legal Medicine, Innsbruck, Austria, four samples (ID-32 muscle, ID-67 liver, ID-74 lung, and ID-97 brain) were analyzed. The frozen tissue samples were divided into halves and one sample of each half was excised from the inner side of the tissue (Harris Uni-Core™ punch tool 3.00 and scalpel). Tissue samples were extracted using the BioRobot EZ1 Advanced XL (Qiagen, Hilden, Germany) and the EZ1 DNA Investigator Kit according to the manufacturer's protocol using the EZ1 Advanced DNA Investigator Card (Large-Volume protocol).

2.2. Real-time mtDNA quantification

The quantity of mtDNA in the extracts was determined with a previously published real-time-based quantification assay (Niederstätter et al., 2007) and modified in (Bauer et al., 2013). The target amplicon size was 134 bp. Instead of hybridization probes, SybrGreen was used as an intercalating dye for PCR product detection and melt curve analysis. Plasmid DNA for a standard curve was prepared as described by using the pCR4-TOPO TA cloning vector of the FastPlasmid Cloning kit for Sequencing (LT). Plasmid DNA was extracted with the Fast Plasmid MiniKit (5Prime GmbH, Hamburg, Germany) according to the manufacturer's protocols and quantified using the NanoDrop1000 (Thermo Scientific, Hamburg, Germany). Reactions were run in a 5-μL volume on an AB7500 with 7500 System SDS software v1.4 (LT).

2.3. Screening assay

All samples were screened for heteroplasmy by amplifying a 1269 bp fragment covering the whole control region (CR) (16024-576) followed by five sequencing reactions covering the whole amplimer (Brandstätter et al., 2007). PCR reactions were performed in 10-μL volumes containing 1x Advantage 2 PCR Buffer (Clontech, BD Biosciences, Palo Alto and Mountain View, CA, USA), 200 μM of each dNTP (Bioline, Luckenwalde, Germany), 0.2 μM of each primer L15900/H00599 (Biomers.net, Ulm, Germany), 1x Advantage 2 Polymerase Mix (Clontech), and 50 pg mtDNA. Reactions were cycled with an initial denaturation step at 95 °C for 2 min followed by 32 cycles of 95 °C for 15 s, 56 °C for 30 s, and 72 °C for 90 s and a final extension step at 72 °C for 10 min. Amplicons serving as template for sequencing and minisequencing were purified enzymatically by addition of 2 μL ExoSap-IT (USB, Cleveland, OH, USA) followed by incubation at 37 °C for 30 min and 80 °C for 15 min.

The BigDye™ v1.1 Terminator kit (LT) was used for direct sequencing in final volumes of 5 μL consisting of 1x BD v1.1 Terminator Ready Reaction Mix, 5xBD Dye Terminator Sequencing Buffer, 0.2 μM primer (L15989, L16268, L00015, L00314, H00484) and 1 μL of PCR product. Cycling was performed according to the manufacturer's protocol. Sequencing reaction products were purified using the DyeEx 96 column plates or the DyeEx 2.0 columns (Qiagen) according to the manufacturer's recommendations. Capillary electrophoresis was run on either a 3130*xl* or a 3100*Avant* Genetic Analyzer (LT) with Foundation Data collection Software v3.0 or v2.0. The resulting sequences were aligned to the rCRS (Andrews et al., 1999) using Sequencher 4.9 and 5.1 software (GeneCodes, Ann Arbor, MI, USA).

If heteroplasmy was suspected, DNA extraction from another portion of the same tissue was repeated and DNA was amplified with another combination of PCR primers (L15851/H00639). The use of different primer pairs additionally excludes a preferential amplification due to a single base mutation within one of the two primer sets. Sequencing was performed with primers selected to cover the position of interest in forward and reverse directions (cf. Online Resource 1).

Questionable positions were additionally examined by minisequencing and cloning experiments and exemplarily samples investigated by MPS.

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