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Mitochondrial DNA mutations in RRF of healthy subjects of different age

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Abstract

To obtain information on the mechanisms responsible of the generation of ragged red fibers (RRF) during aging, we analyzed the mitochondrial genotype of single skeletal muscle fibers of healthy individuals having an age comprised between 45 and 92 years. The sequencing of the D-loop region showed many sequence changes with respect to the Cambridge reference sequence (CRS), in both RRF and normal fibers. These changes were more abundant in RRF and their number increased between 50 and 60, and 61 and 70 years and then remained approximately constant. The analysis of the sequence changes showed that each subject contained one or more changes associated to RRF in positions of D-loop region that either do not change or that change very rarely. In general the same type of RRF-associated change was not found in more than one individual; exceptions were changes in positions 189, 295, 374 and 514, detected in 20–50% of analyzed subjects. In particular the A189G age-associated mutation was found only in old individuals and prevalently in RRF. Sequencing of other two mtDNA regions showed no relevant changes in the 16S/ND1 region and two RRF-associated original mutations, G5847A and A5884C, in two very conserved positions of $tRNA^{Tyr}$. These results indicate that each subject has its own pattern of RRF-associated mutations in both coding and non-coding region of human mtDNA.

Keywords: Human skeletal muscle; Ragged red fibers; mtDNA; Point mutations; Aging

1. Introduction

Human mitochondrial DNA (mtDNA) is a circular double-stranded molecule of 16569 bp; it codes for two rRNAs, 13 polypeptides and 22 tRNAs. The non-coding region, the so-called D-loop region, is about 1.1 kbp long in human, it is the most variable part of the genome and contains regulatory signals for replication and transcription [2,5]. MtDNA lacks histones and has a low-efficient repair system; this makes the molecule very vulnerable to mutations which can become critical in those tissues (postmitotic tissues as muscle and brain) that do not regenerate and are highly dependent on aerobic metabolism. In addition, mtDNA transmission pattern is very peculiar, since it is characterized by a prevalent uniparental (maternal) inheritance, by the persistence in the same cell, as well as in the same mitochondrion, of different forms of mtDNA molecules (heteroplasmy) and by the change of the proportion of mutant mtDNA during cell division (mitotic segregation) [12,19,22]. Many reports associate a wide range of diseases with mtDNA mutations. The mutations are present at a high proportion (from 50 to

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90%) in the affected tissues (mainly post-mitotic) and can be either large scale rearrangements (from few hundreds to thousands of bp) or point mutations affecting protein-coding or tRNA genes [22,24,35]. Mitochondrial DNA abnormalities, although at an extremely low level, occur also in healthy subjects: several large scale deletions and point mutations have been found to accumulate with age in many tissues including skeletal muscle, heart and central nervous system [7,9,15,24–25]. These alterations are accompanied by subtle changes at morphological and biochemical level. The analysis of genotypic and phenotypic alterations on a large number of human skeletal muscle biopsies from healthy subjects of different age [28–29,31] has shown the presence of a small amount (about 1%) of fibers without cytochrome oxidase activity (COX^- fibers). The almost totality of these fibers reacted intensively with the stain of succinate dehydrogenase (SDH) and were classified as ragged red fibers (RRF). These are fibers with an abnormal subsarcolemmal mitochondrial proliferation, considered markers of mitochondrial suffering and found also in many mtDNA-related diseases [3–4,12,20,30,38]. While the analysis of RRF mtDNA in patients suffering from several types of mitochondrial diseases showed the presence of large deletions or point mutations [26–27,30], information on the mitochondrial genotype in RRF from healthy subjects is still rather limited. Recently, Cao et al. [6] found deleted mtDNA molecules associated with ragged red regions of individual muscle fibers from aged rats, suggesting a clonal expansion of deleted mitochondrial genomes in these fibers.

The mechanism accounting for the high number of mitochondria present in RRF is not known. It is possible that as a consequence of a mitochondrial damage due to a pathogenic mutation, the cell tries to compensate for the reduced oxidative potential by increasing the number of mitochondria. Alternatively, the mutations might affect bases crucial for the control of mtDNA replication, so that their change might cause a replicative advantage to the mutated genomes, resulting in their accumulation independently on local cellular energy status or metabolic demand [38]. To get insights on this topic, we determined the sequence of three mtDNA regions in individual skeletal muscle fibers COX^+/SDH normal (COX^+/SDHn), COX^+/RRF and COX^-/RRF. The regions analyzed were the D-loop region; the region 16S/ND1, encompassing the 3′ end of the 16S, the tRNA $^{Leu(UUR)}$ gene and the 5′ end of ND1; and the *tRNA* gene cluster region, containing also the L-strand replication origin and the 5′ end of the *COX* I gene. The results obtained show a large extent of changes in the D-loop region, that are more abundant in mtDNA from RRF. The changes included known polymorphisms, found in all types of fibers, and changes in invariant or rarely variant positions, found prevalently in RRF. We also detected two changes in the 16S/ND1 region and 14 changes in the tRNA gene cluster, two of which, found only in RRF, might be pathogenic.

2. Materials and methods

2.1. Muscle samples

Fifteen human skeletal muscle biopsies (200 mg) from *Vastus lateralis* were obtained with informed consent from healthy Italian patients undergoing routine orthopedic surgery. All subjects had normal physical activity levels and they presented normal medical histories and normal findings on physical, hematological, and biochemical examinations, without clinical or morphological evidences of mitochondrial alterations. The protocol of this study was approved by the Ethical Committee of the "G. D'Annunzio" University of Chieti. Muscle specimens were immediately frozen in isopentane cooled by liquid nitrogen and stored in liquid nitrogen until analysis.

2.2. Single fiber laser microdissection (LMD) and DNA extraction

Serial 16 microns-thick transverse sections from frozen muscle biopsies were cut with the cryotome (HM 505, E-Microm), mounted on polylysine-coated glass slides and stained for COX activity [36], for SDH activity [13,21], and for both activities. Sections were viewed on a Zeiss transmitted light microscope to detect fibers with normal COX activity (COX^+), without COX activity (COX^-), normal SDH activity (SDHn), and hyperreactive SDH activity (RRF). To isolate single fibers, 16 microns-thick transverse sections from the same samples were mounted on Pen foil slides (Leica Microsystems) and stained for COX and SDH activity. Tissue sections were dried for 2 h at 42 °C and viewed on a Leica DM LA upright microscope. Single fibers were cut with the Leica Application Solution Laser Microdissection (AS LMD) and allowed to fall by the force of gravity into capture PCR tubes, located underneath the slide. Fibers were incubated with 25 μl of Proteinase K Extraction Solutions from Pico Pure DNA Extraction Kit (Arcturus) at 65 °C for 3.5 h, followed by heat inactivation of the Proteinase K at 95 °C for 10 min.

2.3. Amplification and sequencing of mtDNA from single skeletal muscle fibers

To sequence the D-loop region, a primary PCR was performed amplifying a 2.1 kbp region of mtDNA from the single cell lysate with primers 15.3 For (15331–15350)–0.8 Rev (836–815). The reaction was performed in a 50 μl volume containing 1× PCR buffer (50 mM Tris–HCl, 15 mM ammonium sulfate pH 9.3, 2.5 mM $MgCl_2$, 0.1% Tween 20), 0.2 mM dNTPs, 0.6 μM primers, 2.5 U JumpStart-REDAccuTaq LA DNA Polymerase (Sigma–Aldrich) and 4 μl single cell lysate. Reaction conditions were 94 °C for 2 min and 35 cycles of 94 °C for 30 s, 58 °C for 30 s and 68 °C for 1.5 min. The final extension proceeded for 8 min. To increase the yield and the specificity of the PCR product, the primary amplification-product (0.5 μl) was used as template