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Aging-associated mitochondrial DNA mutations alter oxidative phosphorylation machinery and cause mitochondrial dysfunctions

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ABSTRACT

Our previous study generated a series of cybrids containing mitochondria of synaptosomes from mice at different ages. The following functional analysis on these cybrids revealed an age-dependent decline of mitochondrial function. To understand the underlying mechanisms that contribute to the age-related mitochondrial dysfunction, we focused on three cybrids carrying mitochondria derived from synaptosomes of the old mice that exhibited severe respiratory deficiencies. In particular, we started with a comprehensive analysis of mitochondrial genome by high resolution, high sensitive deep sequencing method. Compared with young control, we detected a significant accumulation of heteroplasmic mtDNA mutations. These mutations included six alterations in main control region that has been shown to regulate overall gene-expression, and four alterations in protein coding region, two of which led to significant changes in complex I subunit ND5 and complex III subunit CytB. Interestingly, a reduced mtDNA-encoded protein synthesis was associated with the changes in the main control region. Likewise, mutations in ND5 and CytB were associated with defects in assembly of respiratory complexes. Altogether, the identified age-dependent accumulation of mtDNA mutations in mouse brain likely contributes to the decline in mitochondrial function.

1. Introduction

The mammalian mitochondrial genome is a circular double strand DNA with about 16.5 kb in length. Mitochondrial DNA (mtDNA) mainly consists of encoding genes except for about 1.1 kb major non-coding region (NCR). The mtDNA contains 37 genes that encodes 13 proteins for the respiratory complexes (i.e. ND1, ND2, ND3, ND4, ND4L, ND5, and ND6 in complex I, CytB in complex III, COX-I, COX-II, COX-III in complex IV, and ATP6, ATP8 in complex V), and 22 tRNAs and two rRNAs involved in protein synthesis of mitochondria. The major NCR of mammalian mtDNA generally contains heavy-strand promoter (HSP), light-strand promoter (LSP), conserved sequence blocks (CSB), hypervariable segments (HVS), termination-associated sequence (TAS), as well as the classical origin of heavy-strand replication (OH). NCR contains elements necessary for expression and replication. The terms 'NCR' and 'D-loop' are frequently used interchangeably in the literature, although the D-loop region does not span the entire NCR [1]. MtDNA usually has higher mutation rates than nuclear DNA due to its location close to the site of reactive oxygen species (ROS) production, the lack of histone proteins for protection, and the lack of a comprehensive DNA repair system [2]. Each cell contains high copy number of mtDNA, which can be existed in heteroplasmy with both wild type and mutant mtDNA, or in homoplasmy with identical mtDNA. Pathogenic mtDNA mutations are usually heteroplasmic [3,4], whereas neutral polymorphisms in mtDNA are generally homoplasmic.

The roles of mtDNA mutations and oxidative stress in aging have

Abbreviations: BN-PAGE, blue-native polyacrylamide gel electrophoresis; CSB, conserved sequence blocks; CRS, cambridge reference sequence; c, random coil; CI, Complex I; CIII, complex III; CV, complex V; DMEM, dulbecco's modified eagle medium; e, extended strand; FBS, fetal bovine serum; HSP, heavy-strand promoter; HVS, hypervariable segment; h, alpha helix; HVR1, hypervariable region 1; LSP, light-strand promoter; mtDNA, mitochondrial DNA; MRI, magnetic resonance imaging; Mito-Seq, mitochondrial sequencing; NCR, non-coding region; OH, origin of heavy-strand replication; PCR, polymerase chain reaction; P, probability; ROS, reactive oxygen species; RC, respiration chain; SDS, sodium dodecyl sulfate; sub CI, sub complex I; sup CIII, super complex III; sub CV, sub complex V; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; SNV, single nucleotide variation; TAS, termination-associated sequence; t, beta turn; TAS, termination-associated sequence

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been explored over the years. The mitochondrial free radical theory of aging, which assumes mtDNA mutations cause genotoxic oxidative stress, which in turn creates more mutations, has been one of the central hypotheses in the field for decades [5]. In the past few years, however, the evidence on the origin and evolving of such age-related mtDNA mutations has been conflicting [4,6–10].

To explore the role of mitochondria in aging, we generated a series of cybrids containing mitochondria of synaptosomes from mice of different age groups [11]. The unique feature of cybrids is its identical nuclear genomic background among different cybrids, which allows direct elucidation of the role of mitochondrial genome in specific biological process. We found that the overall mitochondrial function in cybrids of the old group significantly decreased compared with young group. Specifically, several cybrids carrying mitochondria derived from synaptosomes of mice at 26 months old showing severe respiratory deficiencies [11].

To further understand the genetic origins for the severe respiratory deficiencies, we interrogated mtDNA in three aging cybrids and one young cybrid as normal control using deep sequencing in order to detect mtDNA mutations with low abundance. Then, using these three aging cybrids as our model system, we further investigated related changes in the structure of the oxidative phosphorylation machinery and the implications on respiratory function.

2. Materials and methods

2.1. Cybrids

Cybrids containing mitochondria of brain synaptosomes from mice at different ages were generated by us as described [11]. Briefly, synaptosomes were isolated from the brains of female C57BL/6 mice aged 6, and 26 months old (representing young, and old group), and synaptosomes from six mice of each group were mixed for a pool. Mitochondria enclosed by cell membrane were isolated from homogenized synaptosomes by centrifuging in density gradient. The mtDNA-less ρ^0 cell line was a derivative of mouse cell line LL/2. The cybrids were generated by mixing ρ^0 cells with the isolated mitochondria in PEG. The cybrids containing synaptosome mitochondria were selected based on the pyrimidine auxotrophy of the ρ^0 cells. Each cybrid cell line was expanded as a clone. To exclude cybrids without mtDNA, mouse mtDNA specific PCR was used to screen cybrids that failed to grow in medium containing galactose. Three cybrids (O25, O49 and O77) from the old group, which found to have severe respiratory deficiencies in our previous study [11], were used as aging cybrids, and one cybrid from the young group with normal mitochondrial function was taken as control. Cybrids were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Invitrogen).

2.2. Detecting of mtDNA mutations by deep sequencing in cybrids with severe mitochondrial dysfunction

2.2.1. PCR amplification of mouse mtDNA

Four pairs of primers as described [12] were used for the PCR amplification: *Mlu*I-mus-B and *Bsp*EI-mus-C for fragment A (1663–6234, 4572 bp); *Bsp*EI-mus-D and *Sph*I-mus-E for fragment B (5943–10829, 4887 bp); *Sph*I-mus-F and *Bgl*II-mus-G for fragment C (10701–15395, 4695 bp); *Bgl*II-mus-H and *Mlu*I-mus-A for fragment D (15200–1846, 2946 bp). The above four fragments span the entire mouse mtDNA, which were taken as deep sequencing templates. High-fidelity long-range PCR was performed by Phanta® EVO HS Super-Fidelity DNA Polymerase using whole cell DNA of cybrids as templates. The following parameters were used for the PCR reactions: 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 s, 62 °C(fragment A) or 66 °C(fragment B, C, D) for 1 min, 72 °C for 5 min, and finally 72 °C for 10 min.

2.2.2. Sequencing data

Sequencing reads mapped to the mouse mitochondrial genome in the 1000 Genomes Project phase 1 data were downloaded from the 1000 Genomes Project data server. The PCR products of the entire mouse mtDNA were sequenced using Illumina Hiseq2500 platform by Research Center of Joy Orient Translational Medicine in Beijing.

2.2.3. Computational pipeline for calling heteroplasmy and polymorphism

Briefly, sequencing reads retrieved from the 1000 Genomes Project data server were remapped to the mouse genome, both nuclear and mitochondrial genomes, using GSNAP [13]. Only reads uniquely mapped to the mitochondrial genome were recorded to minimize the complications of NumtS [14]. Data were further filtered and defined "usable sites" based on the following three quality control criteria: (i) Phred quality score ≥ 20 for used bases; (ii) 30 × coverage of qualified bases on both positive and negative strands; (iii) 95% samples satisfy criteria i and ii. A candidate heteroplasmic site was defined with the following two criteria: (i) the raw frequency for the minor allele is no < 1% on both strands; and (ii) all alleles have support from at least two reads on each strand.

2.2.4. Bioinformatics analysis

The conservation of mtDNA nucleotide was analyzed via Clustal X for all species of mouse, rat, which can be found in Genbank, and human [15], including *Mus musculus* NC_005089 (the sample of this study), *Mus musculus* domesticus AB042432.1, *Mus musculus* molossinus NC_006915, *Mus terricolor* NC_010650.1, *Mus musculus* musculus NC_010339.1, *Rattus exulans* NC_012389.1, *Rattus rattus* FJ355927.1, *Rattus leucopus* NC_014855.1, *Rattus sordidus* NC_014871.1, *Rattus fuscipes* NC_014867.1, *Rattus tanezumi* NC_011638.1, *Rattus praetor* NC_012461.1, *Rattus villosissimus* NC_014864.1, *Rattus tunneyi* NC_014861.1, *Rattus lutreolus* NC_014858.1, *Homo sapiens* neanderthalensis AM948965.1, *Homo sapiens* × 93334.1, *Homo sapiens* D38112.1, *Homo sapiens* [Mbuti] AM711903.1 and *Homo sapiens* AF346992.1. The conservation of mtDNA-encoded amino acid was analyzed via mtSAP Evaluation Database for 61 mammalian species (http://mtsnp.tmig.or.jp/mtsnp/search_mtSAP_evaluation_e.html). The functional effect of mutations was predicted via Provean v1.1.3 (http://provean.jcvi.org/about.php) for mouse and Polyphen2 (http://genetics.bwh.harvard.edu.pph2/) for human. The secondary structure of protein was predicted via SOPMA (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html), and the tertiary structure of protein was predicted via software Discovery Studio 4.1 Visualizer.

2.3. Examination of mtDNA-encoded protein synthesis by [^{35}S] pulse-labeling assay

To measure the mitochondrial protein synthesis, pulse-labeling experiments with [^{35}S] methionine were performed according to protocol described previously [16]. About 2 × 10^6 cells were plated in 10-cm dishes, incubated overnight, washed with methionine-free DMEM, and then incubated for 7 min at 37 °C in 4 ml of the same medium containing 100 μg/ml of the cytoplasmic translational inhibitor emetine. Thereafter, [^{35}S] methionine [0.2 mCi (1175 Ci/mmol)] was added, and the cells were incubated for 30 min. The labeled cells were lysed in 1% sodium dodecyl sulfate (SDS). The protein samples quantified with Bradford method (Bio-rad, Hercules, CA) were electrophoresed through a SDS-polyacrylamide gel (15–20% exponential gradient).

2.4. Analysis of respiratory complex assembly by blue-native gel electrophoresis

Mitochondria were isolated according to procedures described previously [16]. Mitochondrial protein concentration was measured by Bradford method. Blue-Native PAGE (BN-PAGE) [17,18] was used for the separation of respiratory complexes on 4–13% gradient