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Ultradeep mapping of neuronal mitochondrial deletions in Parkinson's disease

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ABSTRACT

Mitochondrial DNA (mtDNA) deletions accumulate with age in postmitotic cells and are associated with aging and neurodegenerative disorders such as Parkinson's disease. Although the exact mechanisms by which deletions form remain elusive, the dominant theory is that they arise spontaneously at microhomologous sites and undergo clonal expansion. We characterize mtDNA deletions at unprecedented resolution in individual substantia nigra neurons from individuals with Parkinson's disease, using ultradeep sequencing. We show that the number of deleted mtDNA species per neuron is substantially higher than previously reported. Moreover, each deleted mtDNA species shows significant differences in sequence composition compared with the remaining mtDNA population, which is highly consistent with independent segregation and clonal expansion. Deletion breakpoints occur consistently in regions of sequence homology, which may be direct or interrupted stretches of tandem repeats. While our results support a crucial role for misannealing in deletion generation, we find no overrepresentation of the 3′-repeat sequence, an observation that is difficult to reconcile with the current view of replication errors as the source of mtDNA deletions.

1. Introduction

Somatic mitochondrial DNA (mtDNA) mutations accumulate in postmitotic cell populations with increasing age and, if high enough levels are reached, can cause dysfunction of the respiratory chain (Bua et al., 2006; Itoh et al., 1996; Sciacco et al., 1994). In the brain, the dopaminergic neurons of the substantia nigra are particularly susceptible to the accumulation of mtDNA deletions, and evidence suggests that these play a role in the pathogenesis of Parkinson's disease (PD) (Dölle et al., 2016). We have previously shown that mtDNA copy number regulation is impaired in dopaminergic substantia nigra neurons of individuals with PD, resulting in gradual takeover by the deleted mtDNA molecules and depletion of the wild-type population (Dölle et al., 2016).

The vast majority of mtDNA deletions in dopaminergic substantia nigra neurons are localized in the major arc of the mtDNA, that is, the region between the origins of replication of the heavy and light strands. Mapping of these deletions has revealed similar qualitative characteristics, including location and size, in both elderly healthy controls and individuals with PD and even monogenic mitochondrial disorders causing multiple mtDNA deletions (Bender et al., 2006, 2008; Reeve et al., 2008; Samuels et al., 2004). This similarity across different biological states and disorders suggests a common molecular process underlying the formation of mtDNA deletions in both health and disease. The mechanisms involved remain however unclear.

The dominant theory is that deletions arise spontaneously at microhomologous sites of short base repeats, due to errors of replication (Shoffner et al., 1989) and/or repair (Krishnan et al., 2008; Shoffner et al., 1989), and subsequently undergo clonal expansion, gradually displacing the wild-type population (Kowald and Kirkwood, 2014). Currently, an active subject of debate, the factual mechanism behind the expansion remains elusive.

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Because mtDNA is constantly turned over and contains multiple sites of tandem repeats, new deletions would be expected to arise continuously, forming a heterogeneous pool of different molecule populations in postmitotic cells. Previous studies in isolated neurons have indeed revealed the presence of a multitude of different mtDNA deletions, with only a few—generally a single—expanded species in each cell. We hypothesize that if the spontaneous generation of mtDNA deletions is ongoing also during postmitotic tissues, each neuron should contain a pool of low-frequency deleted mtDNA species alongside any other dominant mtDNA population.

The mechanisms behind deletion formation and clonal expansion are distinct, and their effects are difficult to discriminate within the experimental data. Previous studies of mtDNA deletions in single neurons have used Sanger sequencing of cloned deleted species (Bender et al., 2006) or gel-separated polymerase chain reaction (PCR) products (Reeve et al., 2008). These methodologies are constrained to the most abundant, clonally expanded mtDNA molecules, whereas background deletions at low heteroplasmic frequencies, which may include newly formed deletions, remained undetected. Owing to consumption of sequencing reads by the few dominant mtDNA variants, sequencing at low depths essentially dilutes the signal stemming from deletional events in favor of the signal from the mechanism of expansion.

Here, we characterize the spectrum of major arc mtDNA deletions at the highest resolution to date by ultradeep sequencing (UDS) of the region in 17 single dopaminergic substantia nigra neurons from 2 individuals with PD. We show that each neuron contains a large and heterogeneous pool of low-frequency mtDNA deletions in addition to 1–4 abundant species. Most of them are located in previously undocumented breakpoints and specifically in areas of direct repeats. In addition, our findings confirm the notion of clonal expansion of somatic deletions by showing that there are small but significant differences in heteroplasmy between any specific deleted mtDNA subpopulation and the rest of neuronal mtDNA population.

2. Material and methods

2.1. Samples

We chose to characterize mtDNA deletions in dopaminergic neurons from individuals with PD. These cells have been shown to contain particularly high levels of deletion enabling us to characterize in depth even low levels of heteroplasmy.

Frozen substantia nigra tissue was obtained from 2 individuals with clinically and pathologically confirmed PD from the Norwegian ParkWest study, a prospectively followed population-based cohort, which has been described in detail (Alves et al., 2009). Brain tissue was collected at autopsy, snap-frozen in liquid nitrogen and stored at −80 °C. The pars compacta of the substantia nigra was identified based on localization, morphology, and neuromelanin pigmentation. Neuromelanin-positive (dopaminergic) neurons were collected from the ventrolateral tier (A9 area) using laser microdissection, lysed overnight and used in downstream PCR analyses as previously described (Dölle et al., 2016).

These studies were approved by the Regional Committee for Medical and Health Research Ethics, Western Norway (REK 131/04 and REK 2010/23). Informed consent was obtained from all subjects.

2.2. Mitochondrial DNA amplification and UDS

To assess mtDNA major arc deletions, we performed PCR amplification and UDS of a 9-kb region (revised Cambridge Reference Sequence 5855-14857) in each of 20 microdissected neurons from the 2 PD patients. Amplification of each sample was performed in a 50 μL reaction using 5 μL of cell lysate as template, forward primer 5′-AGATTTACAGTCCAATGCTTC-3′ and reverse primer 5′-AAGGAGTGAGCCGAAGTTTC-3′. To minimize PCR-introduced sequence variation, we used Prime-STAR GXL DNA Polymerase (reported error rate $= 1 \times 10^{-5}$, Takara Bio Inc). Thermal cycling comprised 1 cycle at 92 °C for 2 minutes and 35 cycles of 92 °C for 10 seconds, 56 °C for 15 seconds and 68 °C for 10 minutes, and 68 °C for 7 minutes. PCR products were quality controlled by agarose gel electrophoresis using 5 μL sample and quantitated via Picogreen fluorometric assay using 2 μL sample at 1:100 dilution. Samples showed concentrations in the range of 10–75 ng/μL. For the UDS, samples were normalized in 50 μL of nuclease-free water using 0.30–1.00 μg of stock DNA material and sonicated on Covaris LE220 (Covaris) to a target insert size of approximately 400 bp. Libraries were prepared using the Genomic Services Laboratory standard whole-genome library preparation protocol with 1.8X cleanup to retain all fragments ≤400 bp insert size and quality controlled by qPCR (KAPA SYBR FAST qPCR; Kapa Biosystems, Inc). Products were sequenced using paired-end 125-nt sequencing on the Illumina HiSeq 2500 v4 chemistry and generating ∼1 Gb of data per sample. Sequencing was performed at HudsonAlpha Institute for Biotechnology (Huntsville, AL).

2.3. Mitochondrial DNA plasmid sequencing

To control for deletion artifacts introduced by sequencing or PCR, we sequenced a 1.7-kb fragment comprising the D-loop (m.16223–16834), the mitochondrial gene *MT-RNR2* (m.2782–3092) and a fraction of the mitochondrial gene *MT-ND4* (m.11977–12055), which had been cloned in a pcDNA3.1 plasmid. The cloned fragment was sequenced directly and after PCR amplification using the same conditions and technical specifications as the single-neuron samples. PCR amplification of the fragment was performed using forward primer m.16223-16244 and reverse primer m.12545-12527, which produced a 1655-bp amplicon.

2.4. Preprocessing of next-generation sequencing data

Raw FASTQ files from the provider were quality trimmed using Trimmomatic v0.36 (Bolger et al., 2014). Primer adaptors were removed using cutadapt v1.11 (Martin, 2011) with the primer

Table 1
Depth of coverage

Sample	Individual	Depth (mean ± SD)
1	1	237,887 (±82,690)
2	1	309,763 (±85,520)
3	1	372,144 (±86,775)
4	1	410,528 (±99,921)
5	1	299,983 (±79,331)
6	1	304,879 (±73,852)
7	1	223,377 (±47,657)
8	1	187,968 (±129,727)
9	1	71,923 (±16,808)
10	1	387,912 (±120,148)
11	1	496,002 (±170,126)
12	2	248,939 (±63,982)
13	2	314,808 (±67,655)
14	2	234,936 (±51,647)
15	2	289,951 (±65,325)
16	2	307,276 (±164,184)
17	2	101,330 (±65,002)
Plasmid	-	243,481 (±13,822)
Plasmid (PCR)	-	941,893 (±357,742)

Sample depth of coverage calculated outside of deleted areas.
Key: SD, standard deviation.