Reduced mitochondrial DNA copy number is a biomarker of Parkinson’s disease

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ABSTRACT
Like any organ, the brain is susceptible to the march of time and a reduction in mitochondrial biogenesis is a hallmark of the aging process. In the largest investigation of mitochondrial copy number in Parkinson’s disease (PD) to date and by using multiple tissues, we demonstrate that reduced Parkinson DNA (mitochondrial DNA mtDNA) copy number is a biomarker for the etiology of PD. We used established methods of mtDNA quantification to assess the copy number of mtDNA in n = 363 peripheral blood samples, n = 151 substantia nigra pars compacta tissue samples and n = 120 frontal cortex tissue samples from community-based PD cases fulfilling UK-PD Society brain bank criteria for the diagnosis of PD. Accepting technical limitations, our data show that PD patients suffer a significant reduction in mtDNA copy number in both peripheral blood and the vulnerable substantia nigra pars compacta when compared to matched controls. Our study indicates that reduced mtDNA copy number is restricted to the affected brain tissue, but is also reflected in the peripheral blood, suggesting that mtDNA copy number may be a viable diagnostic predictor of PD.

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1. Introduction
Parkinson’s disease (PD) is a prototypical age-related neurodegenerative disease, affecting approximately 1% of the worldwide elderly population. The etiopathogenesis of PD is complex and multifactorial, including contributions from environmental and genetic factors, with aging remaining the strongest risk factor.

Sporadic PD was first linked to mitochondrial function in the late 1970’s, when the potent respiratory chain inhibitor 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine was shown to cause parkinsonism in illicit drug users (Davis et al., 1979). Subsequent studies, focusing on complex I-mediated reactive oxygen species formation link PD to a vicious circle of oxidative stress and bioenergetic failure. Moreover, there is compelling evidence that mitochondrial biogenesis, meditated through mtDNA copy number, is important in the development of PD. In addition, cannot fully explain the gross neuronal loss seen in PD. Conversely, comprehensive population studies, focusing on the role of inherited mtDNA variants, have identified phylogenetic clades which reproducibly affect PD risk (Ghezzi et al., 2005; Hudson et al., 2013, 2014; Latsoudis et al., 2008).

The increase of mitochondrial biogenesis, hallmarking by a characteristic increase in cellular mtDNA level (Giordano et al., 2014), is a typical compensatory response to gross mitochondrial dysfunction (Lee et al., 2000) and has been reported in mitochondrial disorders characterized by a complex I defect (Giordano et al., 2014).

Here, we studied the mtDNA content in multiple tissues from PD patients and matched control subjects, comparing mtDNA copy number to cardinal measurements of PD etiology. Our observations indicate that mitochondrial biogenesis, mediated through mtDNA copy number, is important in the development of PD. In addition, our data suggest that mtDNA copy number may be a viable diagnostic predictor of PD.

2. Materials and methods
2.1. Patient cohort
We studied the role of mtDNA copy number in n = 363 peripheral blood samples, n = 151 substantia nigra pars compacta...
(SNpc) tissue samples, and n = 120 frontal cortex tissue samples from community-based PD cases fulfilling UK-PD Society brain bank criteria for the diagnosis of PD (Hughes et al., 1992), comparing the results to matched control samples with no clinical evidence of PD (n = 262 peripheral blood samples, n = 33 SNpc tissue samples, and n = 37 frontal cortex tissue samples). All were of Caucasian origin. All blood samples underwent cognitive assessment, including global cognitive function was assessed using the mini-mental state examination (MMSE) (Roth et al., 1986) and the Montreal Cognitive Assessment (Nasreddine et al., 2005). Cognitive impairment was determined using published criteria (using 1.5 standard deviation as a cutoff) (Yarnall et al., 2014). In addition, samples were genotyped for variants known to affect cognitive function: rs9468 (defining H1/H2, MAPT) and rs429358/ rs7142 (defining APOE1–4) (Nombela et al., 2014) Genotyping was performed by KASP genotyping (LGC, Middlesex, UK).

2.2. MitDNA copy number determination

Total DNA was extracted from blood and tissue samples using standard methods. Quantification of mtDNA was performed in triplicate by multiplex Taqman qPCR amplification of the mitochondrial genes MTND1 and MTND4 and the nuclear encoded gene B2M, using serial dilutions of cloned vectors to ensure reaction linearity and for standard curve quantification, as previously detailed (Grady et al., 2014). MitDNA copy number is expressed as a relative abundance of both MTND1 and MTND4 to the nuclear counterpart, with a correlation of \( r^2 = 0.9941 \) (MTND1:MTND4 derived copy number). This method was preferred to absolute (or standard curve) approaches as the low amount of DNA from microdissected tissues does not allow appropriate standard curve preparation (Giordano et al., 2014). Patient and control samples were randomly assigned to each run to limit run-specific stratification.

2.3. Validation of qPCR results

A random proportion (10%) of samples was replicated from source DNA, with a coefficient or repeat variance estimated as 0.3% (indicating that qPCR results are reproducible).

2.4. Statistical analysis

Data were analyzed using SPSS v22 with data-appropriate tests (detailed in text). Statistical significance was set at \( p \leq 0.05 \).

2.5. Ethics

Local study approval was granted (NRES Committee North-East-Newcastle & North Tyneside 1).

3. Results

We initially investigated the role of mtDNA copy number in peripheral white blood cells (PBCs), identifying a significant reduction in mtDNA copy number in PD cases when compared to controls (case mean = 163 ± 89, control mean = 195 ± 129, Mann-Whitney \( p = 2.86 \times 10^{-4} \), Fig. 1A). This association was independent of age (linear regression PD vs. controls with age as a covariate, \( p = 3.0 \times 10^{-4} \)) and gender (males only, A = 227 vs. U = 129, Mann-Whitney \( p = 5.3 \times 10^{-4} \) and females only, A = 133 vs. U = 132, Mann-Whitney \( p = 4.8 \times 10^{-2} \)). Stratification to affected individuals only failed to find a significant association between PBC mtDNA copy number and phenotypic assessments, including dementia (Mann-Whitney \( p = 6.7 \times 10^{-1} \)), cognitive impairment (Mann-Whitney \( p = 3.9 \times 10^{-1} \)), MMSE score (Spearman correlation \( p = 6.85 \times 10^{-1} \)), Montreal Cognitive Assessment score (Spearman correlation \( p = 1.19 \times 10^{-1} \)), power of attention (Spearman correlation \( p = 8.27 \times 10^{-1} \)), continuity of attention (Spearman correlation \( p = 6.90 \times 10^{-1} \)), and cognitive reaction time (Spearman correlation \( p = 4.96 \times 10^{-1} \); Table 1). Interestingly, we were able to identify a significant association between reduced PBC mtDNA copy number and smoking history (defined as current smoker or smoked <5 y from disease onset) in PD cases (Mann-Whitney \( p = 4.0 \times 10^{-3} \); Table 1); however, we found no direct link between smoking history and PD case/control \( p = 2.9 \times 10^{-1} \).

Subsequent analysis of isolated brain tissue revealed a similar reduction of mtDNA copy number in PD cases, statistically significant in the vulnerable SNpc (Mann-Whitney U = 4.0 \times 10^{-4} \), Fig. 1B), but not in the asymptomatic FC (Mann-Whitney U = 2.91 \times 10^{-1} \), Fig. 1C). Substratification by gender revealed a significant association between SNpc mtDNA copy number in males (males only, A = 100 vs. U = 25, Mann-Whitney \( p = 5.0 \times 10^{-4} \)), but not in females (A = 49 vs. U = 8, Mann-Whitney \( p = 3.97 \times 10^{-1} \)); likely a result of low female sample numbers. We found no significant gender effect in FC mtDNA copy number. Correcting for age, as a covariate in linear regression, supported the association in SNpc (\( p = 4.5 \times 10^{-3} \)) and did not affect the result in FC (\( p = 8.4 \times 10^{-2} \)). In addition, we found no association between SNpc or FC tissue mtDNA copy number and dementia, MMSE, cognitive impairment, or smoking (Table 1). However, again, this is likely a direct result of a smaller sample number.

Fig. 1. Analysis of mitochondrial DNA (mtDNA) content in 3 tissues, showing mean mtDNA copy number per cell (with 95% CI) for PD cases (A, shaded) and matched controls (C, unshaded) from (A) peripheral blood cells (PBC), (B) substantia nigra pars compacta (SNpc), and (C) frontal cortex (FC). Asterisks indicate statistical significance (**p = 10^{-4} and ***p = 10^{-3}). Abbreviation: PD, Parkinson’s disease.
control for is cell loss. Postmortem assessment of neuronal cell death will be anecdotal at best, and although studying isolated postmortem SNpc, we cannot completely rule out glial contamination in PD cases, where a loss of most neurons has occurred. However, glial contamination alone could not explain the gross mtDNA copy-number differences seen between cases and age-matched controls in the SNpc, moreover in the effect in PBCs.

Measuring mtDNA content in cells and tissues is not without problem. Extraction techniques affect template availability and can affect downstream processes, with varying mtDNA sequence affecting specificity. We mitigated these issues, using an established, internally correcting triplex probe-based approach (Grady et al., 2014); however, it is important that this effect is replicated in a similar cohort of PD patients and potentially expanded to a range of neurodegenerative diseases.

Finally, our observations are based on a single experimental series and, although our PBC and SNpc data are complimentary, we cannot wholly determine if this phenomenon is cause or effect or indeed limited to the brain regions under study. To address this further, we recommend longitudinal studies in PD patients, preferably investigating further vulnerable (i.e., cingulate) and unaffected brain regions.

5. Conclusion

Accepting our limitations, our study indicates that reduced mtDNA copy number in PBCs, supported by a subsequent reduction in the affected brain region, is a viable biomarker for the detection of PD.

Disclosure statement

The authors declare that they have no competing financial interests.

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