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Quantitation of a mitochondrial DNA deletion in Parkinson's disease

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A 5 kilobase deletion in mitochondrial DNA (mtDNA) has been reported to be responsible for the specific complex I deficiency in the substantia nigra (SN) of the Parkinson's disease (PD) brain. We have studied mitochondrial respiratory chain function in the SN from control and PD subjects, and analysed mtDNA, extracted from the same tissues, by Southern blot and the polymerase chain reaction (PCR). Quantitation of the levels of the deletion indicate that it does not contribute to the pathogenesis of PD nor to a complex I deficiency but seems likely to be an age-related observation.

NADH CoQ_1 reductase; Parkinson's disease; Mitochondrion; Mitochondrial DNA

1. INTRODUCTION

Idiopathic Parkonson's disease (PD) is a common neurodegenerative disease characterised pathologically by the loss of dopaminergic neurones in the substantia nigra and the presence of Lewy bodies in some surviving neurones. The cause of the cell death underlying PD is unknown. However, the discovery that the neurotoxin, 1-methyl-4-phenyl 1,2,3,6 tetrahydropyridine (MPTP) can induce Parkinsonism in humans [1] has provided a valuable insight into the possible pathogenic mechanism(s) of PD. MPTP is converted to its active metabolite 1-methyl-4-phenylpyridinium (MPP^+), by glial monoamine oxidase B [2], which enters dopaminergic neurones via the dopamine re-uptake system [3]. Once inside the neurones it is actively concentrated into mitochondria where it specifically inhibits NADH CoQ_1 reductase (complex I) activity [4]. The inhibition of complex I leads to a fall in ATP production, which ultimately results in cell death [5].

Complex I activity has been reported to be decreased in the substantia nigra of the PD brain [6] and appears to be anatomically specific to the substantia nigra and disease-specific to PD [7]. These observations have focused attention on the possible role of abnormal mitochondrial function in the pathogenesis of PD.

Complex I dysfunction in PD substantia nigra could be the result of some endogenous or exogenous toxic agent or be due to a mutation in one or more of the nuclear or mitochondrial genes for complex I subunits. Such mutations may be primary or secondary to some process such as oxidative stress, to which mtDNA is particularly susceptible because of its lack of repair enzymes [8]. Complex I is the most vulnerable respiratory chain complex to indiscriminate damage to mtDNA because of its high proportion of mtDNA encoded subunits.

A 5 kilobase (kb) deletion in mtDNA has been found in several neuromuscular diseases associated with mitochondrial dysfunction [9], and is referred to as the 'common deletion'. This deletion has been observed to increase with age [10]. The deletion includes genes for complex I, IV and V subunits as well as numerous tRNAs, and is flanked by 13 bp direct repeats [11] which are thought to render this area prone to deletion via slip replication.

Restriction fragment length polymorphism (RFLP) analysis of mtDNA in PD brain failed to show any major deletions [12,13]. Polymerase chain reaction (PCR) amplification of the common deletion in PD striatum, however, has suggested that there is a 16-fold accumulation compared to control striatum [14]. Such a result, if confirmed, would have important implications for our understanding of the role of mtDNA in the aetiology of PD. We have therefore analysed mtDNA from the substantia nigra of control and PD patients via Southern blotting and PCR.

2. METHODS

2.1. *Biochemistry*

Parkinson patients and controls were matched for age, death to refrigeration and death to autopsy time. The substantia nigra from six patients who had died with idiopathic Parkinson's disease and from six controls was homogenised [6], and protein content and enzyme activities in brain homogenates were determined as previously described [7].

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2.2. *Preparation of DNA*

Brain homogenates were lysed with 0.5% SDS in 75 mM NaCl, 50 mM EDTA, pH 8.0, and digested overnight with 2 mg/ml proteinase K at 37°C. DNA was extracted twice with phenol followed by chloroform: isoamyl alcohol (24:1), precipitated with a 0.1 vol. of 3 M sodium acetate and 2 vol. of absolute ethanol at −70°C for 30 min, centrifuged at 12,000 × *g* for 15 min and the pellets rinsed with ice-cold 70% ethanol. The precipitated DNA was dissolved in 1 mM Tris, 0.1 mM EDTA, pH 8.0.

2.3. *Southern blot analysis*

2 μg of total DNA was digested with *Pvu*II (NBL, UK) with the addition of bovine serum albumin (0.1 mg/ml) and spermidine (10 mM). Digested DNA was electrophoresed through a 0.8% agarose gel, and transferred to nylon membrane (Hybond-N, Amersham, UK) [15]. Prehybridisation and hybridisation, with ^{32}P-labelled human mtDNA, were performed as recommended for Hybond-N. mtDNA was visualised by autoradiography for 24–48h at −70°C.

2.4. *Polymerase chain reaction (PCR) and oligonucleotide primers*

Fragments of mtDNA were amplified from 100 ng of total DNA in a 100 μl reaction mixture containing 0.5 μM of each primer, 2.5 U of Ampli*taq* DNA polymerase (Perkin-Elmer/Cetus), 200 μM of each dNTP, 50 mM KCl, 15 mM $MgCl_2$, 10 mM Tris-HCl, pH 8.3, and 0.05% gelatin. Following an initial 6 min denaturation step at 92°C, the amplification cycles were: 1 min at 92°C, 1 min at 59°C and 1 min at 72°C. PCR was performed for 30 cycles. After amplification, 10 μl of the PCR mixture were electrophoresed through a 1.7% agarose gel containing ethidium bromide (0.5 μg/ml).

The forward (—) primer was located at nucleotides (nt) 8196–8215 (L1) on the light strand and reverse (←) primers were located at nt 8726–8707 (H1) and nt 13524–13506 (H2) on the heavy strand. The L1 and H1 primer pair amplified a 530 bp fragment from wild-type mtDNA. The L1 and H2 primer pair amplified a 351 bp fragment from mtDNA possessing the common deletion. To co-amplify both fragments in the same PCR reaction, 0.3 μM of both reverse primers (H1 and H2) were used with 0.5 μM of the forward primer (L1). Co-amplification reactions were performed in triplicate.

2.5. *Standard curve for the estimation of the common deletion*

The wild-type fragment and the fragment from deleted mtDNA were amplified from control nigral DNA, electrophoresed through a 1.7% agarose gel, recovered from the gel (NA-45 membrane, Schleicher and Schuell) and quantitated spectrophotometrically at 260 nm. To a series of 6 PCR reaction mixtures (as described in section 2.4) 0.1, 1, 10, 50, 100 and 1,000 pg of template for the deleted mtDNA were added along with 1 ng of template for the wild-type mtDNA. The three primers, L1, H1 and H2, were added and the mixtures spiked with 10 μCi [α-^{32}P]dCTP (3.4 pmol). Following co-amplification, fragments were separated by electrophoresis, and the gel slices corresponding to the 0.53 and 0.351 kb fragments were excised. Radioactivity was eluted (Solvable, NEN), counted in a liquid scintillation counter, and corrected for cytosine content.

3. RESULTS

The citrate synthase-corrected mitochondrial respiratory chain enzyme activities are shown in Table I. In Parkinson patients there was no significant difference from controls for complexes II/III and IV activities. Complex I activity, however, was significantly decreased by 42% of the control mean. There was no correlation between complex I activity in Parkinson's disease with L-Dopa treatment, duration of disease and death to freezing of brain (data not shown).

Digestion of total DNA from substantia nigra with *Pvu*II (Fig. 1) revealed the expected restriction pattern for normal mitochondrial DNA, a single 16.6 kb band. The absence of multiple bands or fragment variation would indicate that mtDNA from the substantia nigra of both PD and control subjects does not possess large populations of deleted molecules. Leaving the autoradiograph at −70°C for longer periods failed to show any specific smaller bands.

The more sensitive polymerase chain reaction was used to amplify, from 6 PD patients and 6 controls, a 530 bp fragment from wild-type mtDNA, and 351 bp fragment from mtDNA with the 5 kb common deletion. The 351 bp fragment was identified as a product of deleted mtDNA molecules via nested primer PCR and digestion with four restriction enzymes (data not shown). On the basis of ethidium bromide staining we observed no significant difference in the amount of 351 bp product between PD patients and controls (Fig. 2b). The 530 bp wild-type fragment was amplified to detect variations in the amount of mtDNA in the 100 ng of total DNA used for PCR (Fig. 2a). There was no notable difference in the amount of normal mtDNA between PD patients and controls.

The relative amount of the deleted mtDNA was estimated by co-amplification of both fragments in the same PCR tube as described in section 2. DNA extracted from the substantia nigra from 4 PD and 4 control subjects (subjects PD1-4, C1-4 Table I) was co-amplified and the percentage of mutant mtDNA calculated from the ratio of cpm's in the two fragments corrected for cytosine content. The observed percentages of deleted mtDNA were similar in the PD

Table I

Mitochondrial respiratory chain enzymes corrected for citrate synthase activity

		Complex I (×100)	Complex II/III (×10)	Complex IV (×100)
Parkinson's disease	1	1.79	1.08	1.21
	2	1.94	1.26	0.80
	3	1.90	0.85	0.87
	4	1.89	0.66	0.52
	5	1.83	0.86	0.58
	6	2.81	1.03	0.82
		2.03*	0.96	0.80
Controls	1	3.45	1.12	0.77
	2	3.12	0.86	0.88
	3	2.92	0.94	0.62
	4	4.49	1.08	0.81
	5	4.68	1.33	0.96
	6	3.09	1.23	0.93
		3.53	1.09	0.83

*Significantly lower than in controls: $P = 0.004$, Mann-Whitney *U*-test

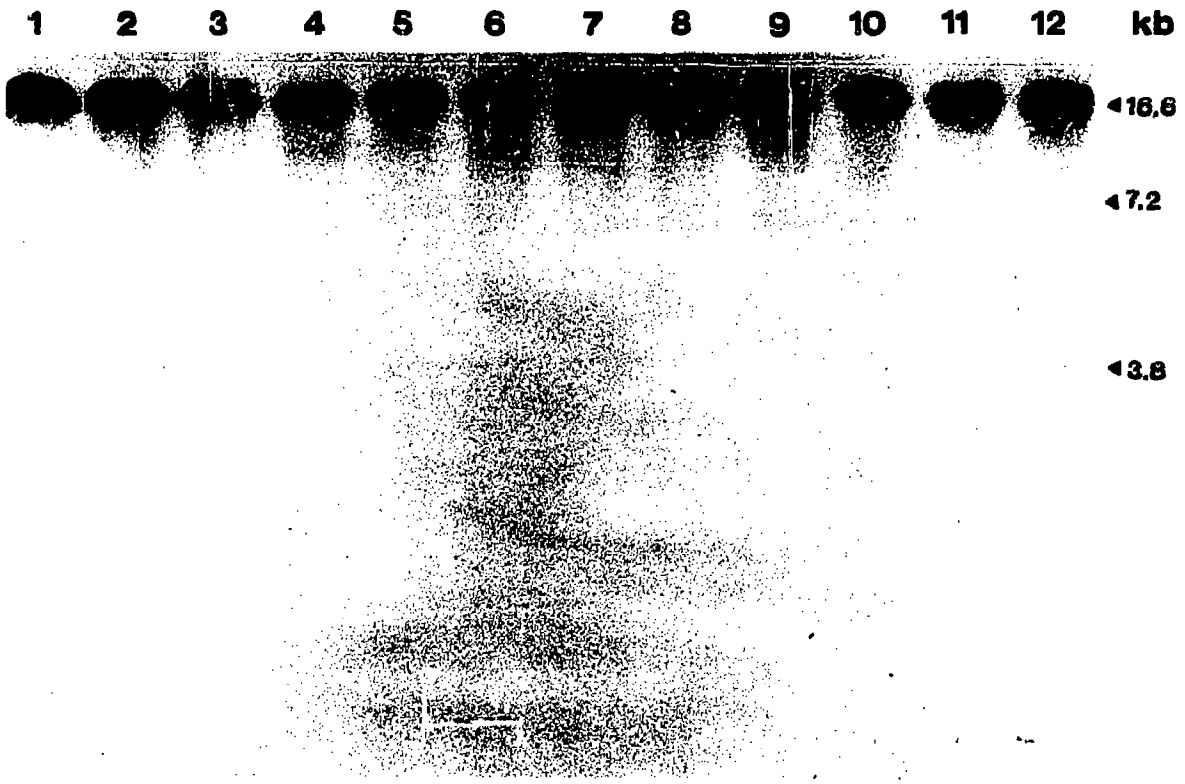

Fig. 1. Southern blot of DNA from the substantia nigra of controls (lanes 1–6) and PD patients (lanes 7–12). DNA samples were digested with *Pvu*II to linearise mtDNA, and probed with normal human mtDNA.

(4.1 ± 0.6%) and control patients (4.1 ± 0.6%). A standard curve was prepared by varying the ratios of the purified templates for the deleted and normal molecule, to determine the differences in PCR efficiency arising from different ratios of normal-to-deleted molecules. As the wild-type:mutant ratio decreased the preferential amplification of the mutant fragment decreased, giving a linear log plot (r=0.97, Fig. 3). Correction for the preferential amplification of the mutant fragment was achieved by interpolation of the observed values from Fig. 3. These show that there is no significant difference in the percentage of mutant mtDNA in the substantia nigra between PD and age- matched control subjects, and that in the aged human brain, either control or PD, approximately 1–2 mtDNA molecules in every 10,000 possess the common deletion (Table II).

4. DISCUSSION

The demonstration of mitochondrial complex I deficiency in the substantia nigra of PD patients has focused attention on the possible contribution that mtDNA mutations may play in the pathogenesis of this disease. mtDNA encodes 7 of the 26 polypeptides of complex I and so an abnormality of mtDNA complex I-encoding genes might be expected to contribute to a functional deficit. The 5 kb common deletion of mtDNA, which is found in a variety of diseases as well as being a normal ageing phenomenon, involves genes that code for four complex I subunits and so is a possible candidate for involvement in PD. Ikebe et al. [16] found the deletion in the striatum from 4 of 5 PD patients and 2 of 6 control subjects. Ozawa et al. [14] quantified the deletion from one of these patients and suggested it was present at a concentration 16-times that of control and that the deleted mtDNA represented 5% of total mtDNA. These results are, however, in contrast to studies that have analysed mtDNA in PD substantia nigra by Southern blotting, in which there was no evidence of any large deletion [12,13].

The results of our study described here demonstrate that the levels of the common deletion present in PD patients and age-matched controls are not significantly different. Furthermore, approximately only 1–2 in 10,000 mtDNA molecules bears the deletion in either group.

The apparent difference between the two sets of data may be due to the methods used. We estimated the proportion of deleted molecules by eluting and counting incorporated radioactivity from the gel bands corresponding to the respective fragments. This avoided potential differences in fragment transfer and hybridisation on membranes, and the non-linearity of absorbance with radioactive signal and exposure time associated with densitometry [17,18]. Also, in our hands, a quantifiable exponential increase in PCR product by

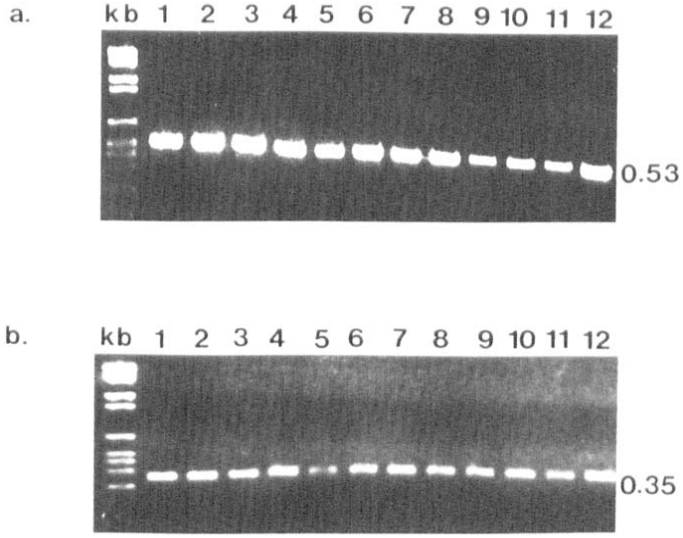

Fig. 2. PCR amplification products of mtDNA from the substantia nigra of PD (lanes 1–6) and controls (lanes 7–12). (a) 0.53 kb product from normal mtDNA. (b) 0.35 kb product from mtDNA possessing the common deletion.

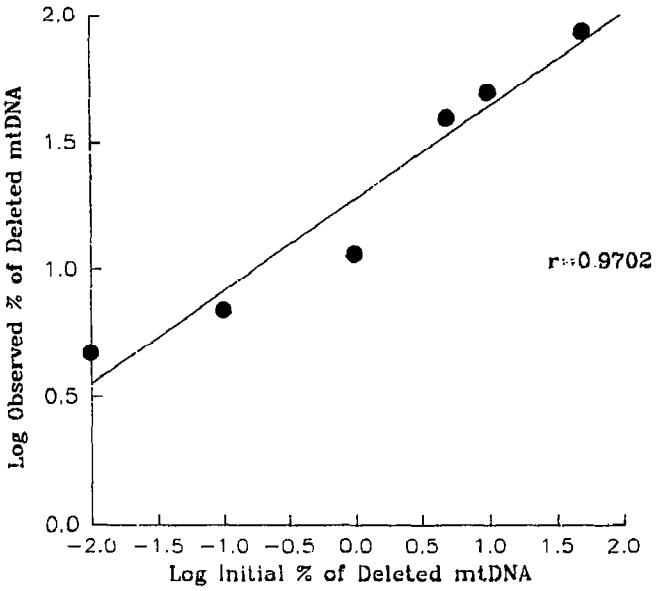

Fig. 3. Standard curve for the estimation of the percentage of mtDNA possessing the common deletion.

terminating reactions at given cycles (12–34 cycles) was not reproducible. This may be due to a combination of (i) rate-limiting reaction components (primers dNTP's, enzyme) (ii) the inhibitory effects of product and pyrophosphates, or (iii) tube to tube variation, as noted by others [19]. The methods described in this report for the estimation of a population of mtDNA can be used for comparative studies.

The difference Ozawa et al. [14] observed between PD and control DNA was probably due to the difference in age between the PD patient (73 years) and control subject (38 years) used in their study. An increase in the presence of the 5 kb deletion with age has been reported in adult heart [10] and human liver [20]. We have also detected increased amounts of the deletion with age in human skeletal muscle showing a 17-fold increase in the common deletion between a 21-year-old and a 78-year-old (manuscript in preparation).

We conclude that the common deletion is not specific to the PD brain and suggest that the presence of the 5 kb deletion is purely an age-related phenomenon, and does not contribute to the pathogenesis of the disease nor to the observed complex I deficiency reported in PD substantia nigra [7]. Whether the accumulation of the deleted genome with age, and a concomitant decrease in respiratory chain function, is a primary or secondary phenomenon in the ageing process remains to be determined.

It is possible that small deletions and/or mutations undetectable on Southern blots may be present and accumulate in mtDNA of Parkinson patients, as is the case in other neurological diseases (mitochondrial encephalomyopathies [21,22] and Lebers hereditary optic neuropathy [23]). Sequence analysis of mtDNA should confirm or exclude the involvement of mitochondrial

Table II

Quantitation of the percentage of mtDNA in the substantia nigra that possess the common deletion

		Age (yrs.)	Observed % (mean ± S.D.)	Calculated % (range)	Mean (calculated) ± S.D.
Parkinson's disease	1	71	4.7 ± 0.35	0.0171–0.0259	0.0216 ± 0.0042
	2	76	4.6 ± 0.50	0.0150–0.0274	0.0214 ± 0.0062
	3	83	3.5 ± 0.38	0.0076–0.0131	0.0097 ± 0.0029
	4	84	3.6 ± 0.18	0.0090–0.0118	0.0106 ± 0.0014
			4.1 ± 0.64		0.0158 ± 0.0066
Controls	1	72	3.5 ± 0.31	0.0076–0.0122	0.0096 ± 0.0024
	2	84	4.5 ± 0.30	0.0161–0.0237	0.0197 ± 0.0038
	3	84	4.6 ± 0.31	0.0171–0.0250	0.0205 ± 0.0041
	4	86	3.7 ± 0.17	0.0105–0.0131	0.0114 ± 0.0015
			4.6 ± 0.56		0.0153 ± 0.0056

genes in mitochondrial dysfunction observed in PD, and therefore its role in the pathogenesis of PD.

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