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Paucity of deleted mitochondrial DNAs in brain regions of Huntington's disease patients

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Abstract

Mitochondrial DNA deletions (Δ-mtDNAs), originally found at high levels in patients with sporadic mitochondrial encephalomyopathies, have also been found to accumulate at extremely low levels during normal human aging, especially in long-lived postmitotic tissues such as muscle and brain. We have now quantitated the amount of one such Δ-mtDNA species, the so-called 'common deletion', in brain regions from patients with Huntington's disease (HD). Surprisingly, we found a marked decrease in the amount of this Δ-mtDNA in the occipital cortex and putamen as compared to age-matched controls; however, no change was found in caudate. Using immunohistochemistry of brain sections, we found no differences in the staining pattern for selected respiratory chain polypeptides between the HD and control tissues. The reduction in the amount of Δ-mtDNAs in HD may be related in part to the astrocytic gliosis in the affected areas, in which the deletion-rich neurons are replaced by relatively deletion-poor astrocytes.

Keywords: Aging; Common deletion; Electron transport chain; GFAP; Glial fibrillary acidic protein; Mitochondrion; mtDNA

1. Introduction

Deletions of mitochondrial DNA (mtDNA) have been associated with progressive external ophthalmoplegia (PEO) and Kearns-Sayre syndrome (KSS), two related sporadic mitochondrial disorders [1–3], as well as with Pearson's marrow/pancreas syndrome, a rare hematopoetic disease [4]. In these disorders, the quantity of deleted mtDNA (Δ-mtDNA) molecules is high, and can be observed easily by Southern blot hybridization analysis. Although the particular species of Δ-mtDNA varies among patients, approximately one-third of all patients harbor the same type of deletion, called the 'common deletion' [5,6], which removes 4977 bp of mtDNA between the ATPase 8 and ND5 genes [7].

The 'common deletion' has also been demonstrated to accumulate during normal human aging, although it is present in extremely small amounts and is detectable only by the polymerase chain reaction, or PCR [8,9]. When the amount of this deletion was compared in different tissues, it was found that long-lived postmitotic tissues, such as muscle and brain, contained much higher amounts of Δ-mtDNA than did the other tissues studied [10,11]. Among the different regions of brain, the caudate, putamen and substantia nigra were found to contain the highest level of Δ-mtDNA [12,13]. The variation in the amount of Δ-mtDNA between the same tissues from different persons of similar age appears to be less than that among tissues within any one individual. It has been suggested that certain environmental insults, such as local free radical production, may influence the accumulation of mtDNA mutations [14–17].

The analysis of mtDNA deletions has been extended to some age-related disorders, such as Alzheimer's disease (AD) and Parkinson's disease (PD), generating controversial results [18]. Ikebe et al. [8] searched for the presence of Δ-mtDNAs in brain tissue of normal subjects and of patients with PD, using PCR. They found that the 'common deletion' was present in brain from all 5 PD patients analyzed (ages 51–77). However, the same deletion was also present in brain from two age-matched controls (age 64 and 73) but not in 4 younger subjects (ages 38–57). Using semi-quantitative methods in a follow-up study, this group estimated that the 'common deletion' was present at a level of about 0.3% in control striatum and about 5% in

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PD striatum [19]. However, the relationship between this level of Δ-mtDNA and the clinical expression of disease remains controversial [20–23].

Unlike Parkinson's disease, which is due mainly to loss of dopaminergic cells, Huntington's disease (HD) is associated with loss of other specific sets of neurons in the basal ganglia, including cholinergic and GABA-ergic neurons [24]. In the study reported here, we have assayed for the presence and amount of the 'common deletion' in selected brain regions from control subjects and from early-onset HD patients, in order to determine if the amount of deleted mtDNA might be increased in this devastating neurodegenerative disease. Surprisingly, we found just the opposite result.

2. Materials and methods

2.1. Materials

Frozen brain tissue samples and paraffin-embedded human postmortem brain tissue were kindly provided by Dr. Edward Bird of the Brain Tissue Resource Center, McLean Hospital (Belmont, Massachussetts). The HD samples were deliberately chosen from 'early-onset' patients in order to minimize the potential confounding effects on our analyses of the known accumulation of mtDNA deletions with increasing age. The ages of the patients (all of whom were 'grade 3' HD patients, as confirmed by postmortem pathological examination) and control subjects ranged from 27–42 years (see Table 1). Restriction enzymes were from Boehringer-Mannheim and New England Biolabs; Taq polymerase was from Perkin-Elmer Cetus; PCR primers were from Genosys; [α-^{32}P]dATP was from DuPont-New England Nuclear; antibody to glial fibrillary acidic protein (GFAP) was from Sigma.

2.2. Isolation of DNA from human tissues

DNA was extracted by the method of Davis et al. [25], as modified. Briefly, 50 mg of tissue were frozen in liquid nitrogen and crushed into powder. Five ml of RSB buffer (10 mM Tris · HCl, 10 mM NaCl, 25 mM EDTA, 1% SDS) containing 1 mg/ml proteinase K was added and incubated at 50° C for at least 2 h. The solution was extracted with isopropanol and centrifuged. After washing with 70% ethanol, the DNA was precipitated with ethanol and then resuspended in 50 μl H_2O. The concentration of total DNA in the samples was approx. 100–500 ng/μl.

Table 1
Amount of 'common deletion' Δ-mtDNA in various brain regions from HD patients

Subjects	Sample #	Sex	Age	Δ-mtDNA/total mtDNA ($\cdot 10^{-4}$) [a]		
				Putamen	Cortex	Caudate
Control 1	b1047	42	M	6.92	0.49	6.43
Control 2	b1366	42	M	1.41	0.15	1.54
Control 3	b0780	27	F	–	0.06	0.41
Patient 1	b1536	36	F	0	0	1.71
Patient 2	b1635	39	M	0.01	0.07	6.68
Patient 3	b1264	37	F	0	0	0.90

[a] A value of zero denotes a level of detection below $0.01 \cdot 10^{-4}$.

2.3. Quantitation of total mtDNA

For quantitation of total mtDNA, total cellular DNA was dot-blotted onto Zeta-blot GT membranes (Bio-Rad) according to the manufacturer's protocol and was hybridized with mp8.M9 (an M13 clone containing mtDNA D-loop sequences from positions 1–740 [7]; a kind gift of Dr. Michael P. King) labelled by primer extension. The amount of total mtDNA in the samples was calculated by comparing the hybridizing signal to that from serial dilutions of known amounts of total HeLa cell mtDNA.

2.4. Generation of an mtDNA standard

Plasmid pC was generated as an internal control for the direct quantitation of the 'common deletion', as described previously [10]. A fragment of deleted mtDNA was amplified by PCR, using primers Xba8289F (mtDNA positions 8273–8305 on the L-strand [7]) and Stu13705B (mtDNA positions 13720–16692 on the H-strand), and the resulting 469-bp PCR fragment was inserted into the PCR1000 cloning vector (Invitrogen). The insert of pC contained sequences from mtDNA flanking the 'common deletion', from positions 8273–8482 (containing $tRNA^{Lys}$ and the 5′-end of the ATPase8 gene) and 13460–13720 (containing sequences from the 3′-end of the ND5 gene), with deletion of the intervening 4977 bp (from positions 8483–13459 inclusive). The DNA sequence of this PCR fragment agreed with the published mtDNA sequence, except for a C → T transition located at nt-8382 within the ATPase8 gene. This change results in the creation of a new cleavage site at nt-8381 for the restriction endonuclease *Ssp*I (AAT|A**T**T; polymorphic site in bold).

Plasmid pC DNA was purified by alkaline lysis followed by glassmilk absorption (Geneclean II kit, Bio101). HeLa cell total mitochondrial DNA was purified by cesium chloride ultracentrifugation [26]. Both HeLa cell mtDNA and pC plasmid DNA were electrophoresed through 1% agarose gels and were quantitated by comparing the ethidium-bromide-mediated UV fluorescence with that generated by serially diluted DNA markers (phage λ digested with *Hin*dIII; Boehringer-Mannheim) as a standard. Quantitation of the standard was confirmed spectrophotometrically.

2.5. Quantitation of the 'common deletion'

A competitive PCR quantitation assay was used to quantitate the 'common deletion' in the samples [10].

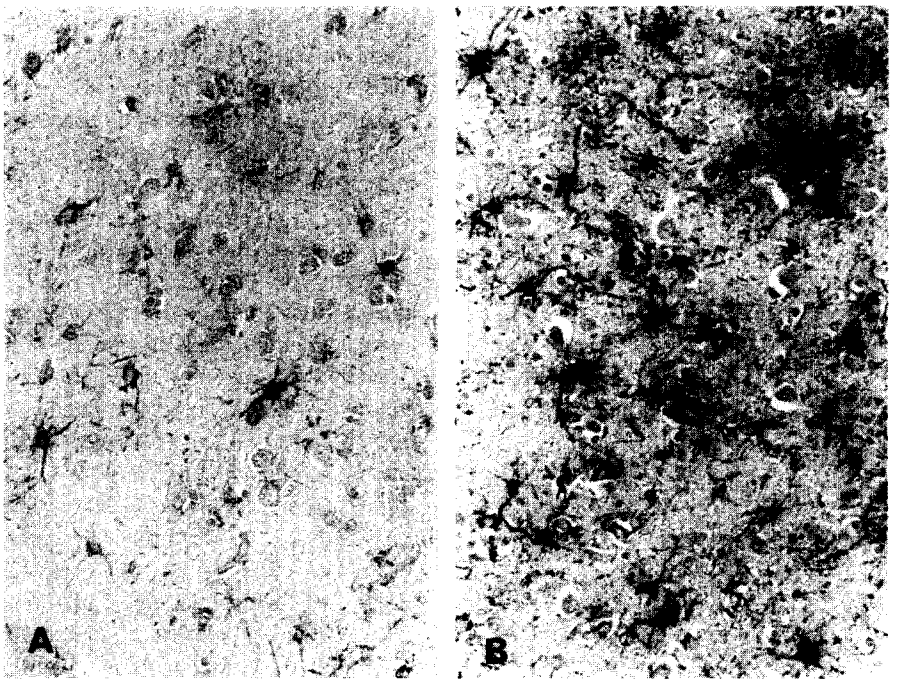

Fig. 1. Immunohistochemical localization of GFAP in brain sections of putamen. (A) Normal control. (B) Patient with HD. Note increased numbers of astrocytes (GFAP-positive cells) in the section from the HD patient.

Serial dilutions of known amounts of the standard plasmid pC were added to the test sample and subjected to PCR using a single pair of primers (Xba8289F and Stu13705B). Digestion with *Ssp*I of the 469-bp PCR fragment derived from amplification of the test mtDNA should result in only two fragments, 293 and 176 bp long, due to cleavage at the single *Ssp*I site normally found in this PCR fragment, at nt-8449. However, the presence of the additional polymorphic *Ssp*I site (at nt-8381) in the PCR fragment derived from amplification of the pC standard should result in cleavage of the 176-bp fragment into 2 smaller fragments, 108 and 68 bp long. By separating and quantitating the amount of radioactivity in the test-specific (176-bp) to the pC standard-specific (108-bp) bands in each sample after polyacrylamide gel electrophoresis, we could calculate accurately the total amount of 'common deletion' present in the test samples [10].

2.6. Western blotting

The autopsied brain tissues were homogenized with a Polytron homogenizer in homogenizing buffer [27]. The amount of total protein in each sample was measured with serially-diluted bovine serum albumin (Sigma) as a standard [28]. The homogenate was mixed with an equal volume of 2 × SDS-PAGE loading buffer and the samples were boiled for 5 min before loading onto a 5–15% SDS gradient gel. Following electrophoresis, the separated proteins were transferred onto a nitrocellulose membrane and glial fibrillary acidic protein (GFAP) was revealed using anti-GFAP antibodies [29].

2.7. Immunohistochemistry

Immunohistochemistry was performed on 5-μm-thick paraffin-embedded sections with the antibodies to subunit 1 of Complex I (ND1) of the respiratory chain, subunit II of Complex IV (COX II), succinate dehydrogenase (SDH), and GFAP, as described [30].

3. Results

3.1. Accumulation of Δ-mtDNAs in brain regions of HD patients

The amounts of 'common deletion' Δ-mtDNA from three regions of brain (putamen, caudate, and occipital cortex) from three early-onset HD patients were compared to the same regions from age-matched control subjects. As shown in Table 1, the level of this Δ-mtDNA species in HD caudate was similar to that in the controls, but was significantly lower than in the controls (approx. by a factor of 1000) in the putamen and in the occipital cortex.

3.2. Immunohistochemistry of selected mitochondrial respiratory chain subunits in HD brain regions

Paraffin-embedded tissues of the brain (caudate, putamen, and occipital cortex) were observed by immunohistochemistry using antibodies to ND1, COII, and SDH. No differences in the distribution or intensities of the immuno-

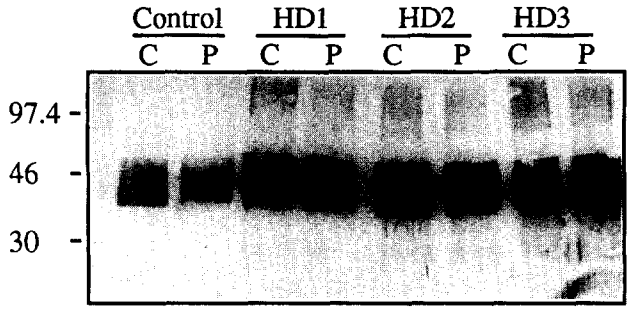

Fig. 2. Western blot analysis of proteins from HD brain regions. Total protein isolated from caudate (C) and putamen (P) from control #1 and from the three HD patients (see Table 1) was subjected to SDS-PAGE followed by Western blot analysis with anti-GFAP antibody. Protein size markers, in kDa, are on the left.

histochemical signal were found between the patients and controls (data not shown).

3.3. Astrocytic gliosis in the basal ganglia of HD brains

Staining of the brain sections from all three regions with anti-GFAP antibody showed that the brains of the HD patients had significant astrocytic proliferation (Fig. 1). This was further confirmed by Western blot analysis of tissue homogenates of the same brain regions: there was a significant increase in the intensity of the signal of polypeptides which migrated in the gel at a position corresponding to GFAP (40–50 kDa), and which displayed immunoreactivity to anti-GFAP antibody (Fig. 2).

4. Discussion

Huntington's disease is an autosomal dominant disorder characterized by the clinical manifestations of chorea, decreased muscular tone, and dementia. While the chorea is thought to be caused by nerve cell death (up to 90%) in the striatum, the impaired cognitive functions and eventual dementia may be due either to the concomitant loss of cortical neurons or to the disruption of normal activity in the cognitive portions of the basal ganglia [31].

We have quantitated the amount of 'common deletion' in selected affected brain regions from early-onset HD patients. Unlike what has been reported in Parkinson's disease (i.e., significant increase [8,19] or no difference [22,23]), we have found that the putamen and cortex of patients with Huntington's disease contained significantly less of this species of mtDNA deletion as compared to the controls. However, we observed no quantitative differences in the caudate.

These results could be due to the involvement of completely different subsets of neurons in the pathogenesis of HD as compared to PD (substantia nigra was not analyzed in this study). The differences we observed in the amount of Δ-mtDNA between the putamen and caudate is unclear, but could be due to structural and functional heterogeneity in the basal ganglia; it has been demonstrated that there are subsets of neurons in this region that differ from each other based on their content of particular substances, such as acetylcholinesterase (AChE) and diaphorase. Furthermore, the neurons in this area appear to receive distinct cortical inputs and also to project differently. In Huntington's disease, these neurons might also be affected differently in the disease process [32]. The most consistent postmortem findings are basal ganglia atrophy with neuronal loss and gliosis in the striatum and globus pallidus. The loss of medium spiny GABA-ergic neurons, with marked reductions in glutamate decarboxylase and GABA, and loss of GABA-ergic projections from the striatum, are the most characteristic and severe abnormalities in HD. This pattern of neuronal loss correlates with the significant reduction in the amount of 'common deletion' Δ-mtDA present in the putamen and cortex of HD brain. Interestingly, we have obtained similar results in some patients with Alzheimer's disease (our unpublished data).

Immunohistochemical examination of basal ganglia and cortex with anti-GFAP demonstrated a significant increase of astrocytes in the affected areas. We suspect that the reduction in the level of deleted mtDNA in HD may be due, at least in part, to the combined effect of neuronal loss and astrocytic gliosis. The limited data on the accumulation of mtDNA deletions indicate that long-lived tissues, such as heart and skeletal muscle, accumulate Δ-mtDNAs to a much greater extent than do tissues that turn over rapidly, such as liver [10]. One would predict that neurons, which are long-lived, would therefore accumulate Δ-mtDNAs to a much greater extent than would glia, which turn over relatively rapidly. Since it is known that neurons are lost in HD and are replaced by glia, the net amount of Δ-mtDNAs would decrease in HD, owing to the combined effects of loss of Δ-mtDNA-rich neurons and gain of Δ-mtDNA-poor astrocytes.

Because of the extensive atrophy observed in the striatum of our samples, there may have been serious variations in the sampling of this region. In order to minimize this problem and to correlate the degree of astrocytosis with changes in Δ-mtDNA levels, we performed the Western blot analysis to detect GFAP on the same tissue samples that were used to perform the quantitation of Δ-mtDNA. Both the putamen and caudate from HD patients contained more GFAP, as measured by Western blotting, as compared to the same regions from normal controls, confirming previous studies [33,34]. For this reason, we believe that our failure to detect the 1000-fold reduction in Δ-mtDNA in HD caudate that was observed in putamen and occipital cortex is real. If true, this finding demonstrates that alterations in the levels of Δ-mtDNAs in HD brain cannot be due merely to replacement of neurons by astrocytes. This conclusion is supported by the fact that a rough calculation of the amount of Δ-mtDNA present in long-lived neurons vs. rapidly-turning-over astrocytes alone

cannot explain a 1000-fold difference in Δ-mtDNA levels between these two cell types. Other factors, which are unknown at present, most likely are involved. For example, the level of deletion might be specific to neuronal subtypes, such that some cells (e.g., dopaminergic neurons) might harbor one level of Δ-mtDNA, while others (e.g., cholinergic and GABA-ergic neurons) might harbor significantly different levels. This supposition is consistent with the finding that Δ-mtDNA levels vary significantly among brain subregions in normal aged individuals, with striatum containing approx. 200-fold more Δ-mtDNAs than cerebellum [12,13]. Interestingly, this difference was observed even in the pathogenic situation, as cerebellum from a patient with sporadic Kearns-Sayre syndrome harboring the 'common deletion' had one-third as much Δ-mtDNAs as did frontal cortex [35].

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References

[1] Holt, I.J., Harding, A.E. and Morgan-Hughes, J.A. (1988) Nature 331, 717–719.
[2] Zeviani, M., Moraes, C.T., DiMauro, S., Nakase, H., Bonilla, E., Schon, E.A. and Rowland, L.P. (1988) Neurology 38, 1339–1346.
[3] Moraes, C.T., DiMauro, S., Zeviani, M., Lombes, A., Shanske, S., Miranda, A.F., Nakase, H., Bonilla, E., Wernec, L.C., Servidei, S., Nonaka, I., Koga, Y., Spiro, A., Brownell, K.W., Schmidt, B., Schotland, D.L., Zupanc, M.D., DeVivo, D.C., Schon, E.A. and Rowland, L.P. (1989) N. Engl. J. Med. 320, 1293–1299.
[4] Rötig, A., Cormier, V., Koll, F., Mize, C.E., Saudubray, J.-M., Veerman, A., Pearson, H.A. and Munnich, A. (1991) Genomics 10, 502–504.
[5] Schon, E.A., Rizzuto, R., Moraes, C.T., Nakase, H., Zeviani, M. and DiMauro, S. (1989) Science 244, 346–349.
[6] Mita, S., Rizzuto, R., Moraes, C.T., Shanske, S., Arnaudo, E., Fabrizi, G., Koga, Y., DiMauro, S. and Schon, E.A. (1990) Nucl. Acids Res. 18, 561–567.
[7] Anderson, S., Bankier, A.T., Barrell, B.G., de Bruijn, M.H.L., Coulson, A.R., Drouin, J., Eperon, I.C., Nierlich, D.P., Roe, B.A., Sanger, F., Schreier, P.H., Smith, A.J.H., Staden, R. and Young, I.G. (1981) Nature 290, 457–465.
[8] Ikebe, S.-i., Tanaka, M., Ohno, K., Sato, W., Hattori, K., Kondo, T., Mizuno, Y. and Ozawa, T. (1990) Biochem. Biophys. Res. Commun. 170, 1044–1048.
[9] Cortopassi, G.A. and Arnheim, N. (1990) Nucl. Acids Res. 18, 6927–6933.
[10] Simonetti, S., Chen, X., DiMauro, S. and Schon, E.A. (1992) Biochim. Biophys. Acta 1180, 113–122.
[11] Cortopassi, G.A., Shibata, D., Soong, N.-W. and Arnheim, N. (1992) 89, 7370–7374.
[12] Soong, N.W., Hinton, D.R., Cortopassi, G. and Arnheim, N. (1992) Nature Genet. 2, 318–323.
[13] Corral-Debrinski, M., Horton, T., Lott, M.T., Shoffner, J.M., Beal, M.F. and Wallace, D.C. (1992) Nature Genet. 2, 324–329.
[14] Miquel, J. and Fleming, J. (1986) in Free Radicals, Aging, and Degenerative Diseases, pp. 51–74, Alan R. Liss, Inc., New York.
[15] Linnane, A.W., Marzuki, S., Ozawa, T. and Tanaka, M. (1989) Lancet i, 642–645.
[16] Ames, B.N., Shigenaga, M.K. and Hagen, T.M. (1993) Proc. Natl. Acad. Sci. USA 90, 7915–7922.
[17] Mecocci, P., MacGarvey, U., Kaufman, A.E., Koontz, D., Shoffner, J.M., Wallace, D.C. and Beal, M.F. (1993) Ann. Neurol. 34, 609–616.
[18] DiMauro, S. (1993) Neurology 43, 2170–2172.
[19] Ozawa, T., Tanaka, M., Ikebe, S.-i., Ohno, K., Kondo, T. and Mizuno, Y. (1990) Biochem. Biophys. Res. Commun. 172, 483–489.
[20] Parker, W.D., Boyson, S.J. and Parks, J.K. (1989) Ann. Neurol. 26, 719–723.
[21] Parker, W.D., Boyson, S.J., Luder, A.S. and Parks, J.K. (1990) Neurology 40, 1231–1234.
[22] Mann, V.M., Cooper, J.M. and Schapira, A.H.V. (1992) FEBS Lett. 299, 218–222.
[23] DiDonato, S., Zeviani, M., Giovannini, P., Savarese, N., Rimoldi, M., C., M., Girotti, F. and Caraceni, T. (1993) Neurology 43, 2262–2268.
[24] Kopin, I.J. (1994) in Basic Neurochemistry, pp. 899–918, Raven Press, New York.
[25] Davis, L.G., Dibner, M.D. and Battey, J.F. (1986) pp. 47–50, Elsevier, New York.
[26] Drouin, J. (1980) J. Mol. Biol. 140, 15–34.
[27] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) in Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
[28] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[29] Towbin, H., Staehlin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[30] Sparaco, M., Hirano, A., Hirano, M., DiMauro, S. and Bonilla, E. (1993) Brain Pathol. 3, 349–354.
[31] Cote, L. and Crutcher, M.D. (1991) in Principles of Neural Science, pp. 647–659, Appleton and Lange, Norwalk.
[32] Albin, R.L., Reiner, A., Anderson, K.D., Dure IV, L.S., Handelin, B., Balfour, R., Whetsell Jr, W.O., Penny, J.B. and Young, A.B. (1992) Ann. Neurol. 31, 425–430.
[33] Borthwick, N.M., Gordon, A. and Yates, C.M. (1985) J. Neurol. Sci. 71, 377–386.
[34] Stevens, C.D., Altshuler, L.L., Bogerts, B. and Falkai, P. (1988) Biol. Psychiatry 24, 697–700.
[35] Shanske, S., Moraes, C.T., Lombes, A., Miranda, A.F., Bonilla, E., Lewis, P., Whelan, M.A., Ellsworth, C.A. and DiMauro, S. (1990) Neurology 40, 24–28.