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## A maternal line study investigating the 4977-bp mitochondrial DNA deletion

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### Abstract

The most frequently reported species of mitochondrial DNA (mtDNA) damage associated with ageing is the 4977-bp ‘Common Deletion’. However, recent observations have raised several issues within the deletion debate namely: the significance of the 4977-bp deletion (CD) as a universal DNA marker of ageing and mitochondrial dysfunction; and the possibility for maternal transmission of deletions in humans. Previous attempts at answering these questions have been limited because many investigations have been cross-sectional studies of unrelated individuals. With the unique feature of the maternal inheritance of mtDNA, our study overcomes some of these limitations by investigating the CD in human maternal lines, which represent 21 families spanning four generations. Using a highly sensitive PCR methodology, we identified the presence of the CD in leukocytes from all 71 individuals (age range—8 months–99 years) including all infants and children ( $n = 15$ ) which in addition were free of any known mitochondrial diseases. This is important because the few reports of the CD in infants have been linked to mitochondrial disease. These results question the significance of the CD as a universal DNA marker of ageing and subsequent mitochondrial dysfunction and provide support for the possibility for maternal transmission of deletions.

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### 1. Introduction

Mitochondria are the major intracellular source of reactive oxygen species (ROS), which cause cumulative damage to cellular components (DNA, RNA, protein and lipids) that is postulated to result in ageing. This process is known as the mitochondrial theory of ageing (Kowald and Kirkwood 2000; Salvioli et al., 2001). The mitochondria are distinct in that they contain their own DNA genome (mtDNA) that has been fully sequenced (Anderson et al., 1981; Andrews et al., 1999). Compared to nuclear DNA, mtDNA is highly susceptible to damage because it is not associated with protective histones, it is continually exposed to high levels of ROS generated by oxidative phosphorylation and although there is base excision repair, there are

none, or limited mechanisms in mitochondria for the repair of DNA damage by the usual process of nucleotide excision (LeDoux et al., 1993; Croteau and Bohr, 1997; Sawyer and Van Houten 1999).

The most frequently reported species of mtDNA damage associated with aging and disease is the CD (Ikebe et al., 1990). Researchers have suggested a role for the CD in both the loss of bioenergetic function with increasing age and the onset of disease (Nagley and Wei, 1998). However, Merrill et al. (1996) demonstrated in brain tissue that the levels of the CD were more associated with chronic hypoxia than aging. Furthermore, Lightowers et al. (1999) have argued that claims for the age accumulation of mtDNA mutations are based largely on non-quantitative data and that no clear functional deficit of mitochondrial respiration has been demonstrated. More recently, this controversy has been fuelled by two observations. First, similar levels of the CD has been identified in human cultured skin fibroblasts derived from fetal tissue compared to unrelated adult donors (Gerhard et al., 2002). Second, a study in human

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Table 1  
Distribution of ages (years) among generations included in the study

Variable	Great Grand-Mother (GGM)	Daughter (D)	Grand-Daughter (GD)	Great Grand-Child (GGC)
Mean (years)	79.6	52.2	31.5	8.6
Minimum	62	36	19	<1
Maximum	99	82	58	29
Standard deviation	10.8	9.6	11.1	8.7
Sample size	18	21	23	18
Number of three generations	6			
Number of four generations	15			
Total # of generations	21			

lymphocytes failed to observe an age-related increase in the CD (Ross et al., 2002).

These observations raise several issues: the significance of the CD as a universal DNA marker of aging and mitochondrial dysfunction; and the possibility for maternal transmission of deletions in humans. Previous attempts at answering these questions have been limited because many investigations have been cross-sectional studies of unrelated individuals. Using the unique feature of the maternal inheritance of mtDNA, our study overcomes some of these limitations through the process of investigating the CD in human maternal lines spanning four generations.

## 2. Materials and methods

The incidence of the CD was investigated in 71 participants from 21 families, ranging in age from 8 months to 99 years (Table 1). A blood sample (< 1 ml) was obtained from a finger prick and stored on a FTA Card™ (Life Technologies). Total DNA (nuclear and mitochondrial) was extracted from the blood samples using the FTA purification protocol (Life Technologies) according to the manufacturer's instructions. Subsequently the total DNA was eluted from the FTA Card™ (following modifications to the manufacturer's instructions) by adding 100 µl of TE to the tubes containing two to three purified paper punches and the samples incubated at 4 °C for 24 h. The DNA concentration in each sample was determined by fluorometry and adjusted to 1 ng/µl. Each PCR reaction was performed in a 25 µl reaction mixture containing; template DNA, one pair of primers (T<sub>1</sub>T<sub>2</sub> or D<sub>1</sub>D<sub>2</sub>) and each deoxyribonucleoside triphosphate (dNTP), MgSO<sub>4</sub>, thermopol reaction buffer, Deep-Vent (*exo-*) DNA Polymerase and Bovine Serum Albumin (BSA) (New England BioLabs Inc). Samples were then amplified with multiplexed primers (Table 2) in a PTC-225 DNA Engine Tetrad Thermocycler (MJ Research, Inc., Waltham, Massachusetts). The volume and concentration of the template DNA, primers, dNTPs, MgSO<sub>4</sub>, buffer, polymerase and BSA are listed in Table 3. The first cycle consisted of a hot start at 95 °C for 5 min. followed by 30 cycles consisting of a denaturation step at 94 °C for 30 s, annealing at 60 °C for 1 min., and primer

extension at 72 °C for 30 s, with a final extension for 10 min. at 72 °C. Wild type mtDNA was determined by amplification of a 177-bp sequence from the highly conserved 12S region (T<sub>1</sub>, 1257–1279nt, and T<sub>2</sub>, 1433–1411nt). Deleted molecules were identified by primers flanking the deleted region, (D<sub>1</sub>, 8416–8437nt and D<sub>2</sub>, 13519–13498nt) which produced a 127-bp product if the deletion was present. Following amplification, for each participant, 4 µl of the wild type and 4 µl of the deleted PCR product were loaded in separate lanes on a 6% polyacrylamide gel. Also, 4 µl of the size/reference standard (pBR322 DNA- Msp 1 Digest) at a concentration of 400 ng, 4 µl of a positive control (DNA from a subject known to carry the CD) and 4 µl of a negative control (TE buffer: 10 mM Tris–HCl, pH 8.0, 0.1 mM EDTA) used in the DNA purification and elution stages were loaded in separate lanes. The gel was run at 140 V, 500 mA for 45 min. After separation, the DNA bands stained with ethidium bromide (50 µg/ml) were visualized by transillumination under UV light. Relative quantification of amplification products was performed using the Kodak Electrophoresis Documentation and Analysis System (EDAS) (Eastman Kodak Company, Rochester, NY) and image analysis software (Kodak 1D Image Analysis Software). The presence of any identified CD was further confirmed by direct DNA sequencing of the 127-bp product.

Table 2  
Oligonucleotide primers used for PCR amplification of the 4977-bp deleted mtDNA and wild type mtDNA

Primer pair	Amplified position 5–3	The length of amplified product (bp)
T <sub>1</sub> T <sub>2</sub> (Wild type)	1257–1433	177
D <sub>1</sub> D <sub>2</sub> (Deleted)	8416–13519	127
T <sub>1</sub> (1257–1279) 5-	TATACCGCCATCTTCAGCAAAC-3	
T <sub>2</sub> (1433–1411) 5-	TACTGCTAAATCCACCTTCGAC-3	
D <sub>1</sub> (8416–8437) 5-	CCTTACACTATTCTCATCACC-3	
D <sub>2</sub> (13519–13498) 5-	TGTGGTCTTTGGAGTAGAAACC-3	

Table 3  
The volume and concentration of the different reagents required for a 25  $\mu$ l PCR reaction mixture

Reagent	Concentration of stock solution	Concentration per reaction	Volume per reaction ( $\mu$ l)
Buffer	10 $\times$	1 $\times$	2.5
MgSO <sub>4</sub>	100 mM	1 $\times$ (2 mM)	0.5
BSA	10 $\times$	1 $\times$	0.25
DNTPs	100 $\times$ (100 mM)	1 $\times$ (1 mM)	0.25
Polymerase	2000 U/ml	0.5 units	0.25
Primers	20 $\mu$ M	0.2 $\mu$ M	0.5
DdH <sub>2</sub> O	N/A	N/A	15.75
Template DNA		1 ng	5.0
Total			25

### 3. Results and discussion

We identified the presence of the 4977-bp deletion in the leukocytes of all 71 individuals from the 21 maternal lines. In agreement with previous studies, the 4977-bp deletion was evident in young and old adults. However more importantly, the 4977-bp deletion was found in all 15 children ranging in age from 8 months to 15 years. A typical example of the results can be seen in Fig. 1. Previous research reporting the CD in a variety of tissues from infants has been associated with mitochondrial disease (Bernes et al., 1993; Bai and Seidman, 2001). In contrast, the infants and children used in our study were free of any known mitochondrial diseases. These results appear to confirm and extend the views of Lightowers et al., (1999) and the recent observations by Ross et al., (2002) and call into question claims that the CD is a universal DNA marker of aging and mitochondrial dysfunction. In addition, there are other deletions that are more abundant than the CD in many tissues (Bodyak et al., 2001). Perhaps some of these deletions are a better marker for ageing studies.

In our study, the observation of the CD in all the age cohorts is likely to be the result of the PCR conditions used, which involved the combination of short PCR products and a highly sensitive polymerase. It is known that the fragment size affects dramatically the amplification efficiency of the CD (Von Wurmb et al., 1998; Ross et al., 2002). In addition, compared to other polymerases such as Taq, Deep-Vent{*exo*-} is a more sensitive polymerase and therefore the use of this enzyme in our experiments ensured the reliable detection of template concentrations as low as 10 fg (Hilali et al., 1997). Recently, Gerhard et al., (2002) were able to detect the CD in cultured skin fibroblasts derived from fetal tissue by increasing the sensitivity of their assay with P<sup>32</sup> end labeled primers. Perhaps the failure in previous studies to detect the deletion in young individuals may have been due to a lack of sensitivity in the PCR assay (Shoffner et al., 1990; Wei, 1992; Nagley and Wei, 1998; Ross et al., 2002). Our study has a distinct advantage compared to the Gerhard study, which looked at the CD in fibroblasts cultured from unrelated individuals. This is because there was however, a large variation in the population doubling

times between individual fibroblast cultures. Importantly, this is known to introduce a large artefactual variation in the level of the common deletion as levels decrease with increasing doubling times (Krishnan and Birch-Machin, 2002; Koch et al., 2001).

These results also suggest the possibility of maternal transmission of the CD. Maternal inheritance of mtDNAs with point mutations has been observed in patients with mitochondrial disease (Shoffner et al., 1990). Although, the transmission of deleted mtDNA has been reported in *Drosophila melanogaster* (Volz-Lingenhohl et al., 1992), it is rarely seen in patients with pathogenic deleted mtDNA (Wallace, 1999; Shoubridge, 2000). Although there are controversial reports of women with mitochondrial pathologies harboring the CD that appears to be transmitted to their children (Bernes et al., 1993) attempts to resolve the inheritance of mtDNA damage have been hampered by a number of problems (Shoubridge, 2000; Brown et al., 2001). Our study overcomes many of these problems by investigating the CD across maternal lines in individuals

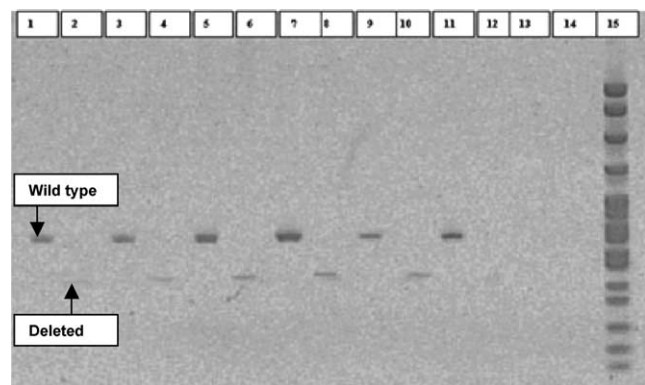


Fig. 1. Electropherogram of PCR products representing wild type and deleted mtDNAs. Lanes 1–8 represent a four generation maternal line. Lane 1: 177-bp fragment from GGC, Lane 2: 127-bp fragment from GGC, Lanes 3 and 4 from GD; Lanes 5 and 6 from D; Lanes 7 and 8 from GGM. Lanes 9–12 represent GD and GGC from another maternal line. Lane 13: negative control for PCR master mix and (primer pair T1/T2), Lane 14: negative control for PCR master mix and (primer pair D1/D2). Lane 15: Size standard pBR322 DNA—Msp 1 Digest, 16 bands ranging from 622 to 767-bp. The 6% polyacrylamide gel was run at 140 V, 500 mA for 45 min.

who did not exhibit any mitochondrial pathologies. Previous works in humans and mice have shown maternal inheritance of a partial duplicated form and its subsequent rearrangement to reproduce pathogenic large-scale deleted mtDNA species (Ballinger et al., 1994; Inoue et al., 2000). In addition, Brenner et al. (1998) reported the presence of mtDNA deletions in both human oocytes and embryos.

Presence of the CD, through the temporal spectrum of these 21 maternal lines, suggests several alternative explanations unrelated to ageing. The CD may be indicative of normal cellular behaviour, associated with apoptosis (Zullo, 2002) or cellular maintenance pathways, irrespective of age. One study suggests that the CD is the result of chronic cellular hypoxia (Merrill et al., 1996). Moreover, there is evidence that the CD is present in normal cells, yet increases in frequency when cellular stress occurs such as that associated with smoking (Lewis et al., 2000). Interestingly, the deletion may be enzymatically excised at tandem repeats which flank this and many others areas where mtDNA deletions occur, suggesting a non-random process.

In a study of maternal lines spanning up to four generations, we have addressed the question of the significance of the CD as a universal DNA marker of aging and mitochondrial dysfunction as well as the issue of the maternal transmission of deletions in humans. Our results support the viewpoint in the current literature, which calls into question the universality of the CD as a marker of the ageing process. In addition, our study contributes to the possibility of maternal transmission of deletions in non-pathogenic humans. The deletion debate continues!

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