Comparison of the relative levels of the 3243 (A→G) mtDNA mutation in heteroplasmic adult and fetal tissues

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Abstract

In this report, levels of the 3243 A to G mtDNA mutation associated with the mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) syndrome were measured in different heteroplasmic tissues of subjects in a kindred including adults with variable clinical phenotypes and a fetus. The relative proportions of mutant mtDNA varied widely (0·03 to 0·67) between identical tissues of the six different subjects and between different tissues of the same subjects. In the one adult for whom sufficient data were available there was an apparent correlation between the distribution of mutant mtDNA and clinical presentation. A woman without neurological symptoms who died prematurely with a cardiomyopathy and lactic acidosis had higher proportions of mutant in heart (0·49, SD 0·02), skeletal muscle (0·56, SD 0·01), and liver (0·55, SD 0·12) than in other tissues studied (for example, kidney, 0·03, SD 0·01). A strikingly different result was found in a 24 week old fetus in whom there was little variation in heteroplasmy in different tissues (average proportion of mutant mtDNA in six tissues, 0·53, SD 0·02). These observations add cardiomyopathy to the growing list of presenting features of the 3243 mtDNA mutation. The unique results from the fetus suggest also that selection pressures acting on either wild type or 3243 mutant mtDNA (rather than variation from replicative segregation of the heteroplasmic mtDNA) may be responsible primarily for the variable levels of 3243 mutant mtDNA in different heteroplasmic tissues of adults.

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The mitochondrial encephalomyopathies include several easily recognisable clinical syndromes,[1–6] as well as a broad range of less well defined syndromes resulting from dysfunction of one or more systems of the body that rely primarily on oxidative phosphorylation for cellular energy production.[7] Different mutations in mtDNA have been associated with many of these.[4 8–10]

MtDNA is found in multiple copies in each mitochondrion and thus in hundreds to thousands of copies per cell.[11–13] Affected subjects are most commonly heteroplasmic (that is, mutant and wild type DNA are both present in the tissue) for disease associated mutations of mtDNA.[14] Although variation in heteroplasmy among matrilineal progeny can be great,[15] variation in heteroplasmy between tissues in the developing embryo is expected to be much less because of the large number of mitochondrial genomes in somatic cells.[11–13 16] However, levels of the MELAS 3243 mutant mtDNA in tissues of adults are also likely to reflect effects of selection and large variation between tissues has been reported in at least two cases.[17 18] Further understanding of these phenomena is important because the proportion of mutant mtDNA in a tissue, and tissue specific thresholds for biochemical expression of defects in oxidative phosphorylation, are believed to be the major factors determining the clinical phenotype.[14]

It would be ideal if the distribution of mutant and wild type mtDNA in a variety of tissues could be followed longitudinally in a heteroplasmic subject, but such an experiment is neither ethical nor practical. As an alternative, we have studied variation in heteroplasmy for the MELAS 3243 mutation between tissues of subjects of different ages. Here we describe levels of this mutation in a range of tissues of different subjects in a three generation kindred and provide data that are, to our knowledge, unique concerning the distribution of this mutation in heteroplasmic tissues of a fetus.

Methods

Material for study was obtained from living subjects after informed consent to procedures was obtained. Use of archival materials was approved by living relatives.

Primary skin fibroblasts were cultured in Ham's F10 medium with 10% fetal calf serum (Gibco BRL, Middlesex, UK). Cells were harvested by trypsinisation after growing to confluence and stored at −70° before extraction for DNA analysis. White blood cells were pelleted after separation from plasma and red blood cells using Dextran 100 and resuspended in phosphate buffered saline. Buccal mucosal cells were harvested by centrifugation (3000 rpm, 15 minutes) of the second of two 25 ml sterile normal saline mouthwashes. DNA from cells was prepared by standard proteinase K-SDS digestion and phenol-chloroform extraction. Freshly frozen tissues

were minced and homogenised, and paraffin embedded or formalin fixed tissues were thinly sliced and extracted three times with xylene, followed by three to six extractions with absolute alcohol before proteinase K-SDS digestion.

For each measurement a segment of mtDNA including bp 3243 was amplified by the polymerase chain reaction (numbered according to the sequence of Anderson *et al*,[19] forward primer, 3130–3149 bp; reverse primer, 3404–3423; cycle 1, 95° for three minutes, 55° for one minute, 75° for 0·5 minutes, cycles 2–34, 95° for one minute, 55° for one minute, 75° for 0·5 minutes, cycle 35, 95° for one minute, 55° for one minute, 75° for 5·5 minutes) using *Taq* polymerase (Boehringer Mannheim GmbH, Mannheim, Germany). *Apa*I (Boehringer) digests (37° for 90 minutes) were performed directly on the PCR products in a reaction mix made from 15 µl PCR product, 2 µl 10 × *Apa*I modifying buffer (125 mmol/l tris-HCl, 38·8 mmol/l $MgCl_2$, 125 mmol/l KCl, pH 7·4), 2 µl water, and 10 U *Apa*I. Digestion products were electrophoresed at 80 V through 3% NuSieve/1% agarose gels and bands were visualised under UV and photographed for analysis. Relative intensities of the 293 (I_{293}) and 180 (I_{180}) bp bands were measured densitometrically from film negatives and the proportion of MELAS 3243 mutant (M) was calculated: $M = 1/(1 + (I_{293}/(1{\cdot}63 \times I_{180}))$. In preliminary trials with standard mixes we confirmed that the proportion of mutant mtDNA could be accurately measured in this way.

Results are expressed as mean values where there are two or more replicates with standard deviations calculated for measurements repeated three or more times. A two tailed Student's *t* test was used to assess statistical significance.

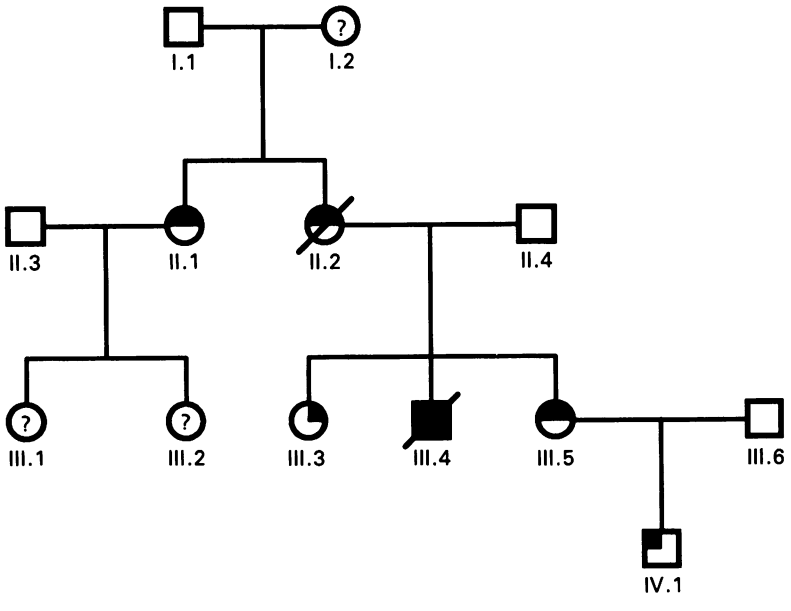

Figure 1 The pedigree of the family. Solid symbol, MELAS syndrome; half shaded symbol, oligosymptomatic; upper right quadrant shaded, asymptomatic; upper left quadrant shaded, phenotype uncertain; ?, not tested or examined.

Results

The family of a boy (III.4) who died at the age of 13 with the MELAS syndrome was studied. In addition to the boy with MELAS, the kindred includes his mother (II.2), who died suddenly at the age of 42 of cardiomyopathy without a history of previously recognised cardiac or neurological disease, two oligosymptomatic women, one with short stature, deafness, and myopathy (III.5) and one with deafness alone (II.1), and an asymptomatic woman (III.3) (fig 1). After delivery of a 24 week stillborn fetus (IV.1) to patient III.5, multiple tissues were obtained for mtDNA analysis. Pathological study of the fetus failed to show any signs characteristic or even suggestive of mitochondrial disease. Archival cultured skin fibroblasts were available from subject III.4 and multiple paraffin embedded specimens obtained from necropsy of II.2. White blood cells from fresh blood samples were obtained from subjects II.1 and III.3.

All members of the kindred tested were heteroplasmic for the MELAS 3243 mutation. The proportion of mutant mtDNA varied from 0·03 to 0·67 between different subjects and different tissues (table). Comparison of the proportion of mutant mtDNA in the same tissue showed differences between subjects, for example, for white blood cells the proportion of mutant mtDNA was 0·39 (SD 0·04) in III.5, 0·19 in III.3, and 0·22 (SD 0·04) in II.1. In II.2 and III.5 there was large variation in the percentage of mutant mtDNA between different tissues. This was particularly striking in II.2, in whom the mutation was near the limit of detection in gut, spleen, and kidney (0·04), although present at significantly higher levels of between 0·49 and 0·56 in cardiac and skeletal muscle and liver ($p < 0{\cdot}001$) (fig 2). A significant difference between the proportions of mutant in cardiac and skeletal muscle was also seen ($p < 0{\cdot}02$), although this was small (difference of means, 0·07).

In contrast to these results, in the fetus IV.1 there was either no variation or only a small variation in the percentage of mutant between different tissues: maximum differences of 0·05 in the proportion of mutant mtDNA were found in comparisons of kidney and cultured fibroblasts or optic nerve ($p < 0{\cdot}01$) (fig 2). It is notable that extra-embryonic tissue (placenta) had a similar proportion of mutant to the several fetal tissues studied.

To determine whether growth in vitro results in a change in the relative proportions of mutant and wild type mtDNA in fibroblasts, a primary culture from IV.1 was repeatedly split and regrown to confluence through approximately 14 generations. There was no measurable change in the proportion of mutant mtDNA over this period (proportion of mutant after 14 divisions, 0·52 (SD 0·02), $n = 4$; primary culture, 0·51 (SD 0·03)).

Discussion

Our kindred shows maternal transmission of the A to G transition mutation at bp 3243 in the mtDNA that is associated with the

Proportions of MELAS 3243 mutation (standard deviation in brackets) in different tissues of subjects in the kindred shown in fig 1. The number of replicates is in square brackets and the range of measurements for each value is given below

	Tissue											
Subject	*Blood*	*Fibroblasts*	*Buccal mucosa*	*Brain*	*Optic nerve*	*Heart*	*Muscle*	*Gut*	*Spleen*	*Liver*	*Kidney*	*Placenta*	*Tissues average*
II.1	0·22 (0·04) [5] 0·14–0·26												
II.2						0·49 (0·02) [3] 0·46–0·51	0·56 (0·01) [3] 0·55–0·57	0·04 [2] 0·03–0·05	0·04 [2] 0·03–0·05	0·55 (0·12) [4] 0·52–0·73	0·03 (0·01) [3] 0·01–0·04		0·29 (0·25)
III.3	0·19 [2] 0·15–0·22												
III.4		0·67 (0·09) [6] 0·58–0·84											
III.5	0·39 (0·04) [6] 0·33–0·42	0·52 (0·06) [4] 0·49–0·62	0·48 [2] 0·44–0·49				0·65 (0·01) [3] 0·65–0·66						0·51 (0·09)
IV.1		0·51 (0·03) [6] 0·46–0·55		0·52 (0·03) [6] 0·48–0·55	0·51 (0·03) [4] 0·47–0·56	0·54 (0·03) [5] 0·52–0·58	0·55 (0·01) [5] 0·54–0·57	0·53 (0·04) [3] 0·47–0·55		0·52 (0·04) [7] 0·44–0·56	0·56 (0·05) [7] 0·52–0·68	0·55 (0·03) [5] 0·51–0·58	0·53 (0·02)

MELAS syndrome.[8] It illustrates again that there is a range of clinical phenotypes associated with the 3243 mutation. Cardiomyopathy has been well described in association with MELAS (found, for example, in one of the first reported cases[20]), although we are not aware that it has been reported as the presenting feature of the 3243 mutation before. It may be relevant that the highest levels of mutant mtDNA were found in organs (heart, skeletal muscle, and liver) whose failure figured prominently in the fatal syndrome of cardiomyopathy and lactic acidosis and almost undetectable levels were found in other tissues studied.

The observation that levels of maternally inherited mutant mtDNA can vary over a broad range between different tissues in heteroplasmic subjects was unexpected[21] given the estimated number of mitochondrial genomes in somatic cells (for example, from no fewer than 500 in fibroblasts to several thousand in myocytes)[11 13] and zygotes (which may have as many as 100 000 mtDNA molecules).[15] If such large numbers of mitochondrial genomes assorted independently, neutral drift theory would predict a relatively slow change in cytoplasmic genotype during somatic cell differentiation and tissue formation.[16] One possible explanation for the large variation seen in patients such as II.2, therefore, is that the number of independently segregating cytoplasmic units of heredity is much smaller than the number of mitochondrial genomes, that is, that mtDNA molecules do not segregate independently into daughter cells as stochastic models have assumed.[16] An alternative explanation for the large variation in heteroplasmy between tissues is that relatively strong, tissue specific selection factors act after replicative segregation in the embryo to amplify preferentially the proportion of either mutant or wild type mtDNA.

There is also a growing body of evidence that selection factors in tissues may cause the proportion of mutant mtDNA in a tissue to change with time as previously shown for mtDNA deletion mutations.[22] First, it seems plausible that there is a relationship between the proportion of mutant mtDNA and mitochondrial dysfunction in any given tissue,[23 24] and clinical studies show that MELAS (which characteristically has onset after birth and normal early development)[1 6] is a progressive disorder. Second, only a subset of the highly oxidative organ systems of the body are most commonly involved in the MELAS syndrome (notwithstanding less frequent pathology in other oxidative tissues, for example, heart). Third, there is a consistently greater proportion of mutant in muscle than in blood[1] and a trend towards relatively greater accumulation in muscle than in blood with age (J Poulton, unpublished observations). Finally, preferential accumulation of the 3243 mutant mtDNA to levels that lead to impairment of mitochondrial polypeptide synthesis has been shown in rho-0 cells repopulated with heteroplasmic mitochondria.[23]

To our knowledge, this is the first report on the distribution of the 3243 mutation between fetal tissues. There has been only one previous report of any mutant mtDNA distribution in the fetus.[25] The contrast between the remarkable similarity in levels of 3243 mutant mtDNA in the broad range of different tissues from the fetus and the large differences seen between some adult tissues suggests to us that tissue specific selection factors affecting mitochondrial genotype are more important than linked segregation of multiple genomes in determining the pattern of tissue heteroplasmy in the adult. As the mechanism and strength of selection may vary for different disease associ-

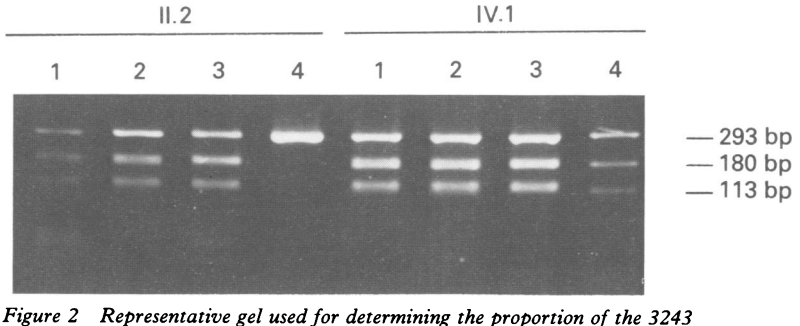

Figure 2 Representative gel used for determining the proportion of the 3243 mutation. The level of heteroplasmy in different tissues from subjects II·2 and IV·1 are shown. A 293 bp mtDNA sequence (between bp 3120 and 3243) was amplified by PCR, digested with ApaI, electrophoresed in a 3% NuSieve/1% agarose gel, then stained with ethidium bromide before photography. The 3243 mutation creates a restriction site for ApaI which is not present in the normal sequence, allowing the proportion of mutant to be determined from the relative intensities of the 180 and 293 bp bands. In the example shown, the tissues analysed were: 1, heart; 2, liver; 3, skeletal muscle; and 4, kidney.

ated mtDNA mutations, the pattern and usual range of variability would probably depend on the nature of the mtDNA mutation together with the genetic background of the subject and environmental effects.

Mitochondrial disease therefore may pose unique problems for genetic counselling, as recently discussed by Harding *et al.*[25] Our data show that heteroplasmy for wild type and 3243 mutant mitochondrial genomes can be maintained in culture. As placental heteroplasmy was similar to that in other tissues, cytoplasmic genotype from chorionic villus cells in a developing fetus might be accurately assessed. However, if the variability in tissue genotypes in adults is determined in large measure after birth, then predictions of clinical phenotype on the basis of fetal genotype may be too unreliable to be useful, even if a direct relationship between the proportion of 3243 mutant and biochemical phenotype of a tissue can be established.

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