Mitochondrial DNA does not appear to influence the congenital onset type of myotonic dystrophy

J Poulton, H G Harley, J Dasmahapatra, G K Brown, C G Potter, B Sykes

Abstract
Neither the maternal inheritance pattern nor the early onset of congenital myotonic dystrophy are fully explained. One possible mechanism is that mitochondrial DNA (mtDNA) mutations might interact with the DM gene product, producing an earlier onset than would otherwise occur. We have used Southern hybridisation to show that high levels of major rearrangements of mtDNA are not present in muscle of five and in blood of 35 patients with congenital myotonic dystrophy. We used sequence analysis to show that no one particular mtDNA morph appears to cosegregate with congenital onset. A minor degree of depletion of mtDNA compared with nuclear DNA was present in the muscle of five patients with congenital DM, but we propose that this is not the primary cause of the muscle pathology but secondary to it. We have not found evidence that mtDNA is involved in congenital myotonic dystrophy.

The DM gene (DMPK) has recently been characterised1 and it is now well established that expansion of a CTG triplet repeat in the 3' untranslated region of the gene is present in patients manifesting the disease. However, both the means by which it causes the adult phenotype and the cause of the in utero onset of congenital myotonic dystrophy (CMyD) remain unexplained.

The expansion may be associated with altered expression of the protein kinase which is encoded by DMPK. There is a rough correlation between the length of the repeat and disease severity,5 but this relationship is not as clear as has been shown for FMR1 in fragile X syndrome.4 Some of the variability may be explained by mosaicism for the expansion: the mean length in peripheral blood leucocytes may not closely reflect that found in muscle.7 Although the length of the expansion in muscle may correlate with severity rather better, the data are limited because biopsy is not essential for diagnosis.

CMyD occurs in about 10% of cases and appears to be quite distinct from severe adult onset DM. As well as earlier onset, there are often phenotypic differences from the typical later onset DM, such as mental retardation and characteristic facial appearance. It is almost always maternally inherited, and is usually associated with large triplet expansions in the mother or child or both.8 However, there is considerable overlap in the length of the expansion in blood between cases with congenital and adult onset.7 Furthermore, the increase in length of the repeat between generations may be more marked with paternal transmission.9 Thus, the magnitude of the fetus’s triplet expansion is not sufficient to explain either the maternal inheritance pattern or the earlier onset of CMyD on its own. A recent study suggests that patients with congenital onset may be distinguished from those with adult onset if both fetal and maternal expansion sizes are taken into account.7 Congenital onset is more likely if both maternal and fetal expansions are large or if either one is very large. This suggests that there may be a determining factor produced by both mother and fetus whose level is influenced by the length of the expansion in both.

We have previously suggested that the maternal inheritance pattern of mtDNA provides a potential mechanism for the phenomena described above.10 Furthermore, an occasional ragged red fibre (a hallmark of mtDNA disease) may be found (0-5%) in patients with myotonic dystrophy,11 as are mtDNA deletions at very low levels.12 This study looked for both qualitative and quantitative differences between patients with congenital or late onset myotonic dystrophy. Minor quantitative changes were found, but we attribute this to the pathological changes inherent in the disease process.

Methods

PATIENT SAMPLES

CMyD was diagnosed using clinical criteria as previously defined.6 Muscle was available from five patients with CMyD (two of whom have been reported before7). DNA was extracted as previously described.13 For Southern analysis, leucocyte DNA was available from 51 families (the majority of whom were ascertained in south Wales), namely, 20 independent families with CMyD, five with early onset DM, 21 with later onset DM, five with minimal disease, and 20 controls (non-consanguineous fathers). Of these, sequence analysis was performed on eight CMyD cases or sibs of cases with congenital onset (including one mother of a case with a 5 kb expansion who was subsequently reclassified as very early onset), 11 cases with...
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Table 1. Expansion size in leucocyte DNA from DM patients and controls

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>No</th>
<th>Alleles measured</th>
<th>Length of smallest expansion (No)</th>
<th>Length of longest expansion (No)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>20</td>
<td>17</td>
<td>85%</td>
<td>5</td>
</tr>
<tr>
<td>Minimal</td>
<td>5</td>
<td>5</td>
<td>100%</td>
<td>0-16</td>
</tr>
<tr>
<td>Adult</td>
<td>21</td>
<td>13</td>
<td>62%</td>
<td>0-18</td>
</tr>
<tr>
<td>CMyD</td>
<td>11</td>
<td>12</td>
<td>92%</td>
<td>2-5-3</td>
</tr>
<tr>
<td>CMyD-M</td>
<td>8</td>
<td>8</td>
<td>100%</td>
<td>0-5-1</td>
</tr>
<tr>
<td>Early</td>
<td>5</td>
<td>4</td>
<td>80%</td>
<td>2-5</td>
</tr>
<tr>
<td>Total</td>
<td>71</td>
<td>58</td>
<td>82%</td>
<td>4-0-8</td>
</tr>
</tbody>
</table>

CMyD = congenital onset myotonic dystrophy.
CMyD-M = mother of a child with congenital onset.

adult onset, and four non-consanguineous controls (spouses). Genotypes at the DM locus were obtained as described by Harley et al.1

SOUTHERN ANALYSIS OF mtDNA
Samples were digested with BamHI which linearises wild type mtDNA, electrophoresed in 0-9% agarose gels, transferred to a nylon membrane, and probed with purified mtDNA which had been labelled with 32P-dCTP by hexanucleotide priming16 as previously described.15

TRINUCLEOTIDE EXPANSION
Repeat length was assessed using standard methods,3 and sized by comparison with a standard. If present, smears were noted and sized.

SEQUENCE ANALYSIS
The sequence of hypervariable region I (bp 16 000-16 400) of the large non-coding region of mtDNA was determined in 23 of the blood samples, using direct sequencing of PCR amplified products. Variants were scored when they differed from the Cambridge Reference Sequence (CRS).17 Diversity within each group was estimated by pairwise difference comparisons and the sequences were compared with 1144 sequences from a world wide data set that included 85 Welsh samples from people with at least two native generations (Sykes et al, unpublished data).

Table 2. Sequence haplotypes found in the mitochondrial D loop in 23 samples of leucocyte DNA from 19 DM patients and four representative controls. All variants were transitions compared to the CRS.17 Reference numbers refer to location of mutation minus 16 000 (for example, "192" refers to a c→t transition at bp 16 192)

<table>
<thead>
<tr>
<th>No</th>
<th>Sex</th>
<th>Phenotype</th>
<th>Region sequenced</th>
<th>Variants at</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>Normal</td>
<td>108-400</td>
<td>192,222,261</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>Normal</td>
<td>70-400</td>
<td>188</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>Normal</td>
<td>103-400</td>
<td>193,221,288</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>Normal</td>
<td>97-400</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>MD</td>
<td>79-400</td>
<td>129,145,222</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>MD</td>
<td>107-400</td>
<td>224,311</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>MD</td>
<td>92-400</td>
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</tr>
<tr>
<td>8</td>
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<td>11</td>
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<td>MD</td>
<td>87-189</td>
<td>189</td>
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<tr>
<td>12</td>
<td>F</td>
<td>MD</td>
<td>103-400</td>
<td>134</td>
</tr>
<tr>
<td>13</td>
<td>F</td>
<td>MD</td>
<td>145-400</td>
<td>270</td>
</tr>
<tr>
<td>14</td>
<td>F</td>
<td>MD</td>
<td>109-400</td>
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<td>15</td>
<td>M</td>
<td>MD</td>
<td>143-400</td>
<td>196,304</td>
</tr>
<tr>
<td>16</td>
<td>F</td>
<td>CMyD</td>
<td>108-400</td>
<td>0</td>
</tr>
<tr>
<td>17</td>
<td>F</td>
<td>CMyD</td>
<td>119-400</td>
<td>0</td>
</tr>
<tr>
<td>18</td>
<td>F</td>
<td>CMyD</td>
<td>77-400</td>
<td>213</td>
</tr>
<tr>
<td>19</td>
<td>F</td>
<td>CMyD</td>
<td>100-400</td>
<td>296,304</td>
</tr>
<tr>
<td>20</td>
<td>F</td>
<td>CMyD</td>
<td>92-400</td>
<td>270</td>
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<tr>
<td>21</td>
<td>M</td>
<td>CMyD</td>
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<td>296,304</td>
</tr>
<tr>
<td>22</td>
<td>F</td>
<td>CMyD</td>
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<td>0</td>
</tr>
<tr>
<td>23</td>
<td>M</td>
<td>CMyD</td>
<td>97-189</td>
<td>189</td>
</tr>
</tbody>
</table>

Quantitation of muscle mtDNA
DNA samples (250-600 ng) from the five patients on whom muscle was available were dot blotted in triplicate using alkaline denaturation. A mitochondrial probe in M13, mt3 (courtesy of Dr M King and Professor G Attardi), corresponding to bp 2578-412217 was labelled with 35S-dCTP by random hexanucleotide labelling. A 1032 bp PCR product corresponding to bp 252-1283 of the arginosuccinate synthetase gene18 was used as a nuclear gene probe and was similarly labelled with 32P-dCTP. Unincorporated dNTPs were removed using a Sephadex G50 column. Hybridisation was at 65°C in Church buffer,19 using 2.5 x 105 cpm/ml and 6 x 104 cpm/ml for 35S and 32P respectively. Filters were washed in 2 x SSC/0.1% SDS at 61°C for 20 minutes and at 63°C for a further 20 minutes and each spot was counted for one hour, six samples at a time, using a flat bed scintillation counter (BetaplateTM Wallac Oy, Turku, Finland). Correction for the spillover between the 35S and 32P pulse height spectra was calculated as previously described.20 Each sample was run on at least two filters, quantified relative to a control, and the average of the readings taken using Chauvenet's criterion for rejection of outliers.

Results
SOUTHERN ANALYSIS
Expansion length was assessed on 41/51 samples of leucocyte DNA from patients with DM. Children with CMyD and early onset DM had expansions ranging from 2.5 kb to a smear of 4-0-8; compared to 0-18 to a smear of 3-8-5 for adult onset. Southern blotting showed no major rearrangements of mtDNA in blood or muscle of patients with CMyD (table 1).

THE DM GENE IS ASSOCIATED WITH LOW/NORMAL LEVELS OF mtDNA IN MUSCLE
While we did not have any muscle from patients with late onset DM, we were able to compare levels of mtDNA relative to nuclear DNA in muscle of five patients with CMyD with normal controls. There was a moderate depletion of mtDNA, compared with age matched controls (figure), but this was not clearly below the normal.

SEQUENCE ANALYSIS
Mean pairwise differences within the groups were not significantly different from the normal Welsh population. One haplotype with trans-
genesis since the majority of respiratory chain subunits are encoded in the nucleus, as are all of the components of their import pathway and enzymes required for their assembly. Mitochondrial (mt)DNA polymorphisms are common, and variant mtDNAs might be neutral unless combined with a nuclear defect such as DM. However, in order to explain the observations, these mtDNA variants would need to cosegregate with the DM gene in order to interact in the embryo: if they were in equilibrium with the rest of the population, a fetus would receive the mtDNA variant as often from an unaffected as an affected mother. An interaction between these genes occurring during the development of the oocyte predicts a model of inheritance pattern very similar to any other maternal factor.

Four possible ways in which mtDNA abnormality and the DM gene might cosegregate were considered in this study.

1. The DM gene might affect mtDNA itself by impairing the fidelity of transmission of mtDNA.
2. The DM gene might alter the quantity of mtDNA in the fetus.
3. Various mtDNA morphs might exacerbate the phenotypic severity of the DM gene, effectively increasing the penetrance of the DM gene.
4. Various mtDNA morphs might improve the survival of fetuses with repeat lengths which might otherwise be lethal.

Heteroplasmy might be present in 1, 3, or 4, particularly if a specific point mutation arose with a high frequency in myotonic families. Alternatively, homoplasmic mtDNA variants might have significant effects in the presence of the DM gene. If so, it might be possible to identify classes of related mtDNA morphs which contained the relevant mutations.

**Discussion**

CMyD appears to be qualitatively different from severe DM with later onset. Several factors have been suggested over and above the DM allele to explain why some patients with apparently identical expansions develop congenital as opposed to later onset DM. These include maternal factors such as imprinting, transmission of a transplacental factor, and mitochondrial inheritance. This study has investigated the role of mtDNA in CMyD, and has not found evidence that it is involved.

Since the human mitochondrial genome was characterised and sequenced in 1981, it has been a clear candidate for causing diseases with a maternal inheritance pattern and associated with defects of the respiratory chain. This is because it encodes polypeptides involved in electron transport and is maternally inherited. The mitochondrial myopathies which have both of these characteristics have been associated with mutations of mtDNA. Heteroplasmy (more than one distinct population of mtDNA in the same person) is rare in health but common in disease, presumably because homoplasy for many of the mutations would be lethal.

How might mtDNA interact with the DM gene to cause CMyD? Multiple interactions between nuclear and mitochondrial genes are known to occur during mitochondrial bio-

**NORMAL LEVELS**

**THE DM GENE DOES NOT APPEAR TO IMPAIR THE FIDELITY OF TRANSMISSION OF mtDNA**

Southern blotting showed no major rearrangements in blood or muscle of patients with CMyD. Thyagarajan et al. looked for point mutations by sequencing the entire mitochondrial genome in two families with congenital myotonic dystrophy. They did not find any point mutation which appeared to be specific or any evidence of heteroplasmy. Investigation of muscle from 13 patients with adult onset myotonic dystrophy using quantitative PCR showed increased levels of mtDNA deletions compared to age matched controls.

However, the level of deletions was too low to detect on Southern blots. Similar levels of deleted mtDNAs have been detected in atrophic muscle in conditions such as inclusion body myositis, aging, and ischaemia (J Poulton, unpublished data), and are probably therefore non-specific.

**THE DM GENE IS ASSOCIATED WITH LOW/ NORMAL LEVELS OF mtDNA**

We have previously shown that skeletal muscle from normal infants and fetuses has low levels of mtDNA compared to nuclear DNA, and...
cross sectional data suggest that the levels increase with age. While there appears to be a distinct group of young patients with reduced cytochrome oxidase activity in muscle, in whom severe mtDNA depletion reflects the primary defect, some patients with other forms of muscle disease (such as ischaemia and inclusion body myositis) may have a moderate degree of depletion. This suggests that mtDNA depletion may be a relatively non-specific response of muscle to various pathological processes. There was a moderate depletion of mtDNA relative to nuclear DNA compared with age matched controls in the muscle of five patients who had CMyD (figure), but this was not clearly below normal. We suggest that this depletion of mtDNA may be attributable to atrophy of type 1 fibres which are aerobic, as these contain more mtDNA than type 2 fibres (which are glycolytic). We conclude that the minor reduction in level of mtDNA relative to nuclear DNA in muscle from patients with CMyD is more likely to be secondary than primary.

(3) and (4) CONGENITAL ONSET MYOTONIC DYSTROPHY IS NOT ASSOCIATED WITH A CLUSTER OF mtDNA MORPHS

Sequence analysis of D loops from patients with CMyD did not show any striking clustering of mtDNA morphs except in the presence of the single haplotype (16 296, 16 304). This was unique to the patient group but was not restricted to the CMyD patients. This is likely to be the result of fairly recent but unrecognised common ancestry, since the majority of the patients were ascertained in South Wales. Otherwise mtDNA morphs defined by the D loop haplotypes were neither more diverse nor remarkable within each group. It is thus unlikely that a long-standing homoplasmic mtDNA mutation plays a role in the congenital onset.

In conclusion, we did not find any support for a role of mtDNA in CMyD. While we did not sequence the entire mitochondrial genome, we believe that our data are sufficient to exclude most simple hypotheses for mtDNA involvement. Thus, neither the maternal transmission of CMyD nor the phenomenon of anticipation are readily explained by the maternal inheritance pattern of mitochondrial DNA (mtDNA). While there are other possible causes for some of the features (such as impaired survival of sperm with large triplet expansions), there is still no unifying explanation.

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