Molecular and functional effects of the T14709C point mutation in the mitochondrial DNA of a patient with maternally inherited diabetes and deafness


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Abstract

A heteroplasmic T to C transition at nucleotide position 14709 in the mitochondrial tRNA glutamic acid (tRNAGlu) gene has previously been associated with maternally inherited diabetes and deafness (MIDD). To investigate the pathogenic mechanism of the T14709C mutation, we have constructed transmitochondrial cell lines by transferring fibroblasts mitochondria from a patient with the mutation into human cells lacking mitochondrial DNA (mtDNA) (rho− cells). Clonal cybrid cell lines were obtained containing various levels of the heteroplasmic mutation, or exclusively mutated or wild-type mtDNA. Measurement of respiratory chain enzymatic activities failed to detect a difference between the homoplasmic mutant and homoplasmic wild-type cybrid cell lines. However, a subtle decrease in the steady-state levels of tRNAGlu transcripts in some mutant clones. Our studies suggest that the T14709C mutation is insufficient to lead impairment of mitochondrial function in homoplasmic osteosarcoma cybrid clones, and that we cannot exclude that the T14709C mutation affects mitochondrial function by a yet unidentified mechanism.

Keywords: Mitochondrial diabetes; Mitochondrial DNA; Point mutation; Transmitochondrial cybrid

1. Introduction

Over the last decade, mutations of the mitochondrial genome have become increasingly recognised as important causes of human disease. Many of these abnormalities take the form of mtDNA rearrangements, but an increasing number of mtDNA point mutations have now been described, affecting protein coding, rRNA or tRNA genes (MITOMAP http://www.gen.emory.edu/mitomap.html).

MtDNA abnormalities, including deletions, duplications and point mutations, are a well-recognised cause of diabetes [1–5]. The most commonly described mtDNA defect in patients with maternally inherited deafness and diabetes (MIDD) resides in the tRNA(Leu(UUR)) gene [2–6], an A to G transition at position 3243 that was first associated with the mitochondrial encephalopathy, lactic acidosis and stroke-like episodes (MELAS) syndrome [7]. Moreover, a point mutation (T14709C) in the mitochondrial tRNA gene for glutamic acid (tRNAGlu) has been identified in some patients with diabetes mellitus [8–11]. In these cases, the T14709C mutation was heteroplasmic and present at high levels in the patient’s muscle and white blood cells. Biochemical studies of muscle mitochondria showed reduced complex I and IV activities [8–10], whilst a correlation was also shown between the level of mutation and the activity of citochrome c oxidase in single muscle fibres [9]. In addition, profound alteration of oxidation process was demonstrated in vivo by 31P magnetic spectroscopy [10].

The development of human cell lines lacking mtDNA (rho− cell lines) that can be repopulated with exogenous
mtDNA by mitochondria-mediated transformation [12] has provided a useful tool to study pathogenic mtDNA mutations, as all cybrids contain the same mtDNA haplotype within the same nuclear background, differing by only the single nucleotide point mutation [13–15]. We have used this methodology to investigate the molecular pathogenic mechanism of the T14709C mutation. Using fibroblasts from a previously characterised patient with this mutation as a source of donor mitochondria [10], we have isolated several clonal cell lines that contained exclusively homoplasmic mutated or wild-type mtDNA and heteroplasmic mtDNA and performed detailed biochemical studies of these cell lines to assess the effect of the T14709C mutation on mitochondrial function.

2. Materials and methods

2.1. Patient

The patient presenting diabetes and severe myopathy was described in detail previously [10]. Briefly, a muscle biopsy revealed ragged red fibers and a decreased activity of respiratory chain complex IV (39% of mean control values). Molecular analysis demonstrated the presence of a heteroplasmic T14709C mutation at a level of 78% mutant load in skeletal muscle and 65% in blood.

2.2. Cell lines and culture conditions

Fibroblasts from skin explant obtained from this patient were cultured in high-glucose DMEM supplemented with 10% foetal bovine serum (FBS), 100 μg/ml pyruvate and 50 μg/ml uridine in 5% CO₂ at 37 °C. The 143B.TK− osteosarcoma cell line and the daughter 143B.206.TK− rho− cells were provided by M. King (T. Jefferson University, Philadelphia, PA, USA). 143B.206 rho− cells, completely depleted of mtDNA by long-term exposure to ethidium bromide, were grown in DMEM containing 4.5 g/l glucose and 110 μg/ml pyruvate, supplemented with 10% FBS (Gibco), 50 μg/ml uridine and 100 μg/ml 5-bromo-2′-deoxy uridine (BrdU, Sigma Immunochemicals, Saint Louis, MO).

2.3. Cell fusion

Transmitochondrial cell lines were obtained using the method of King and Attardi [12]. Rho− cells were collected by low-speed centrifugation, resuspended in DMEM, and counted. Fibroblasts (10⁶ cells) were mixed with an equal number of rho− cells, and culture medium carefully eliminated by centrifugation. Cells were resuspended for 1 min in 0.2 ml of sterile polyethylene glycol 1500 (50% w/v) (Roche Diagnostics, Meylan, France). After cell fusions, screening of complemented cells was performed by growing cells in standard DMEM for 24 h and then in selective medium (pyruvate- and uridine-free DMEM supplemented with 5% dialysed FBS and 100 μg/ml BrdU). After 15 days, about 27 independent colonies were observed and selected in the same medium used for the rho− cells.

2.4. DNA analysis

Total DNA from blood cells and from cybrids cells was prepared using standard protocols [10]. Hair follicles and buccal cells were prepared and subjected to PCR amplification as described [16,17]. For the quantitation of the T14709C tRNA⁸⁸⁰ mutation, a 284-bp fragment was ampli-
fied using the polymerase chain reaction (PCR) method. Conditions were as follows: one cycle at 94 °C for 3 min, followed by 30 cycles at 94 °C for 30 s, 62 °C for 30 s and 72 °C for 30 s; the final extension proceeded for 10 min. Primers for the reaction were 5′ CACGGACTACAAC-CACGACCAATCATA 3′(nt 14682–14708) and 5′ TTACGTCTCGAGTGATGTGG 3′(nt 14966–14947). The underlined position in the first primer represents a modification from the wild-type sequence to create an NdeI restriction site in wild-type mtDNA. The amplification products were digested with NdeI, which cleaves the 284-bp fragment into two fragments of 259 and 25 bp. The T14709C mutation abolishes this NdeI restriction site. To determine the proportion of normal and mutated mtDNA, the amplified fragment was labeled with [α-32P]dCTP in the last cycle to prevent underestimation of the proportion of mutant mtDNA due to heteroduplex formation. Following digestion, the restriction fragments were submitted to electrophoresis through a 15% polyacrylamide gel, the gel was dried onto a support and exposed to radiographic film. The relative proportions of mutant and wild-type mtDNA were quantified by scanning and analysing a shortly exposed X-ray film with the Image 1.45 software.

Nuclear genotypes were characterised by high polymorphic markers analysis. PCR fragments with CA repeats from chromosome 5 (D5S455) were amplified and sequenced using an ABI Prism automated DNA sequencer (Perkin Elmer) [18]. The highly polymorphic repetitive sequence D11S533 present on chromosome 11q was evidenced in the different cell lines, as described [19].

2.5. Respiratory chain enzyme analysis of transmitchondrial cybrid clones

Mitochondrial fractions were isolated from cultured cells and the activities of the individual respiratory chain complexes and the matrix marker citrate synthase were determined spectrophotometrically as previously described [20,21].

2.6. High-resolution Northern blot analysis

Total cytosolic RNA was isolated from 1 to 2 × 10⁶ cells using TRIZOL® Reagent (Life Technologies, UK). Large RNA species were precipitated by the addition of 10 M LiCl, allowing smaller RNAs (5S, tRNA) to be precipitated from the resulting supernatant. Small RNAs (1 μg) were separated through a 13%, 8 M urea denaturing polyacrylamide gel using 1× TBE as running buffer, electroblotted onto GeneScreen-plus membranes (NEN Dupont) in 0.25× TBE and immobilised by UV crosslinking. Regions of mtDNA encompassing the tRNA^{Glu} and tRNA^{Leu(UUR)}
genes amplified by PCR were used as probes for Northern blots. The tRNA\textsubscript{Glu} probe (69 bp) was amplified using the forward primer L14635 (positions 14635–14655). 5'TACTAAACCACACTCAACAG 3' and the reverse primer H14810 (positions 14810–14791) 5'GGAGGGTGGATGAATGAGTGG 3'. The tRNA\textsubscript{Leu(UUR)} probe (75 bp) was amplified using the forward primer L3200 (positions 3200–3219) 5'TATACCCACACCCACCCAAG 3' and the reverse primer H3353 (positions 3353–3334) 5'GCCAGTTAGATTGGTGACAC 3'. Purified PCR products were radiolabelled with [\alpha\textsuperscript{32}P]dCTP (3000 Ci/mmol) (Amersham, UK) by the random-primer method and unincorporated nucleotides were removed by gel filtration through a Sephadex G-50 DNA grade column (Pharmacia, UK). Hybridization was carried out at 42 °C overnight in a solution of 5 × SSPE, 50% formamide, 10% dextran sulphate, 5 × Denhardt's solution, 1% SDS containing 2 × 10\textsuperscript{6} cpm radiolabelled probe. After hybridization, two 15-min washes were performed at room temperature with 2 × SSPE, followed by a 15-min wash at 65 °C with 2 × SSPE and 2% SDS. Blots were subjected to PhosphorImager analysis and the radioactive signal for the tRNA\textsubscript{Glu} probe (69 bp) normalised to that of the tRNA\textsubscript{Leu(UUR)} probe (75 bp) for each sample using ImageQuant software (Molecular Dynamics).

3. Results

3.1. Tissue distribution of the T14709C mutation and respiratory chain activity measurements in fibroblasts

Fig. 1 shows that the proportion of mutant mtDNA was highest in the muscle of the patient (82%) as compared to the proportion found in white blood cells (61%). The T14709C mutation was found in buccal cells (69 %) and in hair follicles (65%) of the patient. Skin fibroblasts cultures were established from the patient carrying the T14709C mutation. Analysis of fibroblasts established from the patient revealed a mutant load of 71%. Respiratory chain enzyme activities determined from mitochondria-enriched preparations [19] showed a reduced complex I activity (57%) and a slightly decreased complex IV activity (72%) in the patient compared to controls.

3.2. Cell fusion and characterization of the cybrid cells

To obtain homoplasmic mutant transmitochondrial cell lines, patients’ fibroblasts were fused with rho\textsuperscript{−} cells fully depleted of mtDNA derived from the 143B.TK\textsuperscript{−} cell line. The growth capacity of the different cell lines in various media was assessed (Fig. 2). The rho\textsuperscript{−} cells exhibited both auxotrophy for pyruvate and uridine and resistance to BrdU, as they were obtained from the thymidine kinase-deficient (TK−) osteosarcoma 143B cell line. Patient’s fibroblasts exhibited BrdU sensitivity and were able to grow in DMEM lacking pyruvate and uridine (Fig. 2). After fusion, selection of cybrids was performed in the selective medium containing BrdU but lacking pyruvate and uridine. After 15 days, we collected 27 transformant cybrid clones. The different clones were able to grow in the selective medium with growth kinetics similar to those of rho\textsuperscript{−} cells in a pyruvate- and uridine-supplemented medium.

We analysed the mtDNA genotype of the 27 cybrids clones by PCR-RFLP analysis. Eleven clones were heteroplasmic ranging from 4% to 95% mutated DNA (Fig. 3A). Nine of 27 clones contained 100% mutant mtDNA, whereas seven were judged homoplasmic for wild-type mtDNA (Fig. 4A). We confirmed the presence of normal amounts of mtDNA in each clone by Southern blot analysis (the mtDNA probe was a 15.6-kb purified PCR product amplified with the Expand Long Template PCR system and the nuclear probe was a 18S probe) (data not shown).

Each clone was subsequently examined to verify that the mitochondrial genotype was identical to the donor fibroblasts by sequence analysis [22,23] and that the nuclear genotype was identical to that of the nuclear parent, the 143B cell line. We demonstrated that the DNA of the wild-type and mutated cybrid clones were homozygous for the
same length polymorphism at the D11S533 locus of their nuclear parent, 143B, which differed from the heterozygous pattern found in the donor patient’s fibroblasts (Fig. 3B). Moreover, the allelic marker pattern D5S455 found in the rho<sup>j</sup> cells was evidenced by sequence analysis of the cybrid cells DNA (data not shown). This clearly indicated that the nucleus in cybrid cells originated from rho<sup>j</sup> cells.

### 3.3. Respiratory chain enzyme analysis of transmitochondrial cybrid clones

The activities of complexes I, II and IV were measured in mitochondrial fractions prepared from four wild-type cybrid clones and five homoplasmic mutant cybrids, and compared to the activity of the mitochondrial matrix marker enzyme, citrate synthase (Table 1). No significant differences in the enzymatic activities between homoplasmic mutant cells and homoplasmic wild-type cybrid clones were observed.

### 3.4. tRNAGlu steady-state levels

To investigate the effect of the T14709C mutation on the processing of tRNAGlu from its precursor, the steady-state level of tRNAGlu<sup>33C</sup> was determined by Northern blot hybridization in the three homoplasmic wild-type and three homoplasmic mutant cybrid clones. The tRNAGlu did not show any obvious size change in the mutant form compared to controls, although there appeared to be a decrease in the quantity of the mature tRNAGlu transcripts in two of the three mutant clones to about 50% of the amount present in the wild-type clones (Fig. 4).

### 4. Discussion

In an attempt to further characterise the pathogenic mechanism of the T14709C tRNAGlu mutation of mitochondrial DNA, we have constructed transmitochondrial cell lines by transferring fibroblast mitochondria from a patient previously shown to be heteroplasmic for this mutation [10], into the 143B.TK<sup>−</sup> osteosarcoma rho<sup>+</sup> cell line. Several clones were isolated that contained either exclusively the wild-type allele (homoplasmic for 14709T) or the mutant allele (homoplasmic for 14709C), and assessed for respiratory chain enzyme activities. No discernible difference in respiratory chain function was noted between the homoplasmic mutant and wild-type cybrids. High-resolution Northern analysis revealed steady-state levels of tRNAGlu transcripts to be subtly decreased in some mutant clones, thus highlighting the limitations of these transmitochondrial cybrids for the investigation of pathogenic mutations.

The T14709C transition was first described in two families in whom the index case presented with an adult-onset myopathy and diabetes mellitus [8,9]; mental retardation and cerebellar ataxia was evident in members of one pedigree [8]. The mutation affects a highly conserved nucleotide flanking the anticodon of the tRNAGlu, a functionally important region of the tRNA structure (Fig. 5). Incidentally, a mutation (A4295G) at the analogous position of the mitochondrial tRNA<sub>Ile</sub> gene has also been documented as causing disease [24]. The T14709C mutation was shown to be heteroplasmic and present at high levels in skeletal muscle. Muscle biopsy of affected individuals revealed mitochondrial accumulations (ragged-red fibres) and a proportion of fibres expressing focal defects of cytochrome <sub>c</sub> oxidase (COX-negative fibres) [8,9]. Biochemical studies in muscle also revealed a defect in the activities of complexes I and IV [8–10], consistent with an impairment of mitochondrial translation. These data, together with the demonstration that COX-negative fibres harboured significantly higher levels of mutated mtDNA than functionally normal, COX-positive have assigned the mutation as pathogenic in accordance with fulfilled accepted criteria [25]. To elucidate the molecular mechanism of any

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**Table 1**

Mitochondrial respiratory chain enzyme activities in transmitochondrial cybrids

<table>
<thead>
<tr>
<th></th>
<th>Wild type, n=4 (mean ± S.D.)</th>
<th>M5</th>
<th>M7</th>
<th>M8</th>
<th>M20</th>
<th>M21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complex I/CS</td>
<td>0.145 ± 0.064</td>
<td>0.089</td>
<td>0.162</td>
<td>0.120</td>
<td>0.078</td>
<td>0.125</td>
</tr>
<tr>
<td>Complex II/CS</td>
<td>0.292 ± 0.103</td>
<td>0.266</td>
<td>0.276</td>
<td>0.193</td>
<td>0.318</td>
<td>0.415</td>
</tr>
<tr>
<td>Complex IV/CS</td>
<td>1.34 ± 0.32</td>
<td>0.821</td>
<td>1.025</td>
<td>2.127</td>
<td>1.230</td>
<td>2.360</td>
</tr>
</tbody>
</table>

Four wild-type and five totally mutated (M5, M7, M8, M20, M21) cybrid clones were analysed. Enzyme activities from mitochondria-enriched preparations are expressed as nanomoles NADH oxidised per minute per unit citrate synthase (CS) activity for complex I, nanomoles DCPIP reduced per minute per unit of citrate synthase for complex II and the apparent first-order rate constant per second per unit citrate synthase for complex IV (× 10<sup>3</sup>). DCPIP = 2,6-dichlorophenol-indophenol; S.D. = standard deviation. Assays were performed in triplicate.
potential pathogenic mutation, however, studying homoplasmic transmissomal mitochondrial cybrids remains very much the method of choice [12–15].

The most puzzling aspect of our results is that the studies of the T14709C mutation in cybrids did not reflect the biochemical defect (partial complex IV deficiency) expressed in mature muscle of our patient, or those seen in previously reported cases [8,10]. We believe this may be due to a number of reasons, not least the fact that the expression of mitochondrial DNA variants is dependent upon a combination of nuclear background and mtDNA genotype. Non-random segregation of pathological and wild-type mtDNA molecules in osteosarcoma and lung carcinoma cells has been observed previously in particular for the A3243G mtDNA mutation [26] and partially duplicated mtDNAs [27]. Moreover, Hao et al. [28] have observed that under selective pressure, some transmissible mitochondrial cybrids containing essentially homoplasmic levels of the G5703A mutation could regain normal mitochondrial function, in a process that appears to be associated with changes in the nuclear background. As cells respond to the presence of a mutation that impairs tRNA function by a variety of compensatory mechanisms, this might explain why essentially homoplasmic cybrid clones exhibited slight differences in the level of tRNA Glu transcripts.

It is reasonable that the primary molecular consequences of the T14709C mutation might be due to a translation defect by impairing the translation of glutamate codons, or to reduced levels of aminoacylated tRNA Glu . Recent studies have investigated this possibility in patients with MELAS due to the A3243G mutation, following the isolation of another mutation (at position 12300) in the anticodon of the mitochondrial tRNA Leu(CUN) gene that was able to suppress both the protein synthesis defect and respiratory impairment of cells harbouring very high levels of mutant mtDNA in a lung carcinoma line [29]. Although the suppressor tRNA is efficiently aminoacylated in the lung carcinoma cell line, aminoacylation of the tRNA Leu(UUR) is severely decreased [15,30]. These data, together with studies in A3243G MELAS biopsy samples that reported decreased amounts of aminoacylated tRNA Leu(UUR) in some, but not all, biopsy samples suggest that the molecular consequences of the mutation can be as diverse in vivo as they are in cultured cells and are dependent upon the nuclear background [31].

The large variation of the clinical phenotype in MIDD associated with the T14709C mutation is striking [8,10]. Although some of this diversity may be accounted for by intra-tissue variability of mutation load, the involvement of additional nuclear factors is likely to contribute to the phenotypic variability of the MIDD disease.

In conclusion, the T14709C mutation currently fulfils all accepted criteria to be documented as pathogenic. It has been detected in several families, is heteroplasmic and is associated with a measurable biochemical defect in patient’s muscle. Our studies indicate that T14709C tRNA Glu mutation is insufficient to lead impairment of mitochondrial function in these homoplasmic osteosarcoma cybrid clones and although reduced levels of mutant tRNAs were observed, this may highlight a limitation of the cybrid cell system for studying pathogenic mtDNA mutations. More extensive work, including the analysis of further cybrids with different nuclear backgrounds, will be required before the exact correlation between clinical symptoms and state level of tRNA Glu is understood.

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References


