Variable penetrance of a familial progressive necrotising encephalopathy due to a novel tRNA\textsuperscript{Ile} homoplasmic mutation in the mitochondrial genome

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Introduction: We present a family comprising a clinically normal mother and two daughters, each with severe encephalopathy with onset in late childhood. A third daughter had died previously of an earlier onset but neuropathologically similar disease.

Methods: Sequence analysis of the entire mtDNA was carried out in muscle, fibroblasts, and lymphocytes of the affected daughters and unaffected mother. Biochemical analysis of individual respiratory chain enzymes was performed on the same tissues, and on several transmitochondrial cybrid clones containing the nucleus of a 143B.206 osteosarcoma cell line and the mutant mtDNA.

Results: Genetic analyses revealed in both daughters and mother the presence of a novel mutation in the tRNA\textsuperscript{Ile} gene of mtDNA, which was homoplasmic in fibroblasts, lymphocytes, and skeletal muscle of the two patients. It was also homoplasmic in fibroblast and skeletal muscle samples of the mother, and approximately 97% heteroplasmic in her lymphocytes. Combined defects of complexes I and IV of the mitochondrial respiratory chain were found not only in fibroblasts of the two probands, but surprisingly also in those of their clinically unaffected mother. The respiratory chain defect segregated in transmitochondrial cybrids containing the nucleus of a 143B.206 osteosarcoma cell line and the mutant mtDNA, indicating that the latter was responsible for the biochemical phenotype.

Discussion: Our results support the concept that homoplasmic mutations in tRNA genes can be responsible for mitochondrial disorders characterised by extremely variable penetrance. Although still unexplained, this phenomenon has important consequences in the nosological characterisation, clinical management, and genetic counselling of mitochondrial disorders.

Mutations in mitochondrial DNA (mtDNA) account for a substantial fraction of mitochondrial disorders, a group of clinical syndromes associated with abnormalities of the terminal component of mitochondrial energy metabolism—that is, oxidative phosphorylation (OXPHOS).\(^1\) Oxidative phosphorylation is carried out by the four enzyme complexes of the respiratory chain (complexes I-IV), plus the ATP synthase complex (complex V). MtDNA contains the genetic information to encode 13 polypeptides, which are essential components of complexes I, III, IV, and V, and most of the RNA apparatus necessary for their in situ translation—that is, 22 transfer RNA (tRNA) and two ribosomal RNA (rRNA).\(^2\) Mutations of mtDNA include large scale rearrangements, which are usually sporadic, and point mutations, which are usually transmitted through the maternal lineage. While large scale rearrangements are invariably heteroplasmic—a state in which both mutant and wild type mtDNA species co-exist in the same cell or tissue—point mutations may be either heteroplasmic or homoplasmic (that is, all copies of the mtDNA carry the mutation), and can affect structural genes or genes encoding the RNA apparatus involved in mitochondrial protein synthesis.\(^3\)

One particularly intriguing feature of OXPHOS disease is the extreme variability of biochemical and clinical presentations. The basis for this variability remains largely unknown. As far as heteroplasmic mtDNA mutations are concerned, the pathogenetic role of a given sequence change can be validated on the basis of rigorous pathogenetic criteria, such as its absolute segregation with the disease, obvious impairment of gene function (for example, frameshift mutations in structural genes), and quantitative correlation of mutation load with clinical and biochemical findings. By contrast, a clear pathogenetic role of homoplasmic mutations has been documented in only a few instances, for example, for mutations in the ND genes which give rise to Leber’s hereditary optic neuropathy (LHON), or for the homoplasmic A1555G mutation in the 12S rRNA gene which is associated with aminoglycoside-induced or spontaneous, non-syndromic maternally inherited hearing loss (MIHL).\(^4\) The pathogenic significance of these mutations has been persuasively demonstrated, mainly based on a consistent association between maternal lineages carrying a given mutation and the presence of family members affected by specific syndromes (such as LHON or MIHL). However, little is known about the genetic and epigenetic factors determining the remarkably variable penetrance of homoplasmic mutations. Likewise, the reason as to why some mutations only affect a specific tissue whilst other mutations affect several organs, or why a particular mutation can produce widely different clinical symptoms in different individuals, remains poorly understood. This scenario is further complicated by the recent report of two homoplasmic point mutations in mt tRNA genes. The first mutation, 4300A\rightarrow G in tRNA\textsuperscript{Ile}, has been associated with hypertrophic cardiomyopathy in different kindreds.\(^5\) The second, a homoplasmic point mutation in the mtDNA encoded tRNA\textsuperscript{Val} gene, was found in a family

Abbreviations: BAEP, brainstem auditory evoked potential; LHON, Leber’s hereditary optic neuropathy; MEP, motor evoked potential; MIHL, maternally inherited hearing loss; OXPHOS, oxidative phosphorylation; PCR, polymerase chain reaction; RFLP restriction fragment length polymorphism; SSEP, somatosensory evoked potentials
Figure 1. Pedigree tree. Affected individuals are represented by solid black symbols. Slashed symbols indicate deceased individuals.

A new pathogenic homoplasmic mtDNA mutation

The mutation is a novel T>C change in mtDNA rRNA10, whose pathogenicity has been proven by biochemical and genetic evidence.

Case report

The family pedigree is shown in fig 1.

The index case (subject II-1) was the third child born to non-consanguineous parents. Pregnancy, birth, and early psychomotor development were normal. The patient was in good health until the age of 16, when she suddenly developed blurred vision, diplopia, headache, vertigo with frequent falls, and generalised weakness and malaise. At the same time she gained more than 10 kg (body mass index = 30 kg/m²) in only a few weeks and was found to be hypertensive with impaired glucose tolerance. The clinical examination disclosed up-beat nystagmus with bilateral gaze evoked horizontal nystagmus and a right VIth nerve palsy. Optokinetic nystagmus was severely disturbed in both directions. General medical and neurological examinations were otherwise normal. Neuropsychological testing revealed no deficits (IQ = 96). The lactate concentration in the cerebrospinal fluid was mildly elevated (2.8 mM, normal values <1.2 mM). Blood lactate and pyruvate were normal both at rest and after an exercise test up to 100 Watts. Abnormal laboratory findings also included impaired glucose tolerance, hyperlipidaemia due to high blood triglycerides, and hyperuricaemia. An electroencephalogram showed mild diffuse slowing (8 Hz) with paroxysms. Electromyography and nerve conduction studies were unremarkable. Brainstem auditory evoked potentials revealed bilaterally delayed P100 latencies. Brain magnetic resonance imaging was normal, as was plasma lactate and creatine kinase. A muscle biopsy failed to show abnormalities.

An elder sister of the index patient (subject II-2) died unexpectedly at the age of 1 year, of respiratory arrest after a minor viral infection. Psychomotor development had been normal until then. In the autopsy report, symmetric spongy degeneration of the brainstem was noted, together with marked telangiectatic vascular abnormalities in the mesencephalon.

The mother of both patients, subject I-2, is a 55 year old woman with no neurological symptoms. Clinical examination, neurophysiological testing (BAEP, SSEP, MEP, nerve conduction studies), and magnetic resonance imaging were normal, as was plasma lactate and creatine kinase. A muscle biopsy failed to show abnormalities.

MATERIALS AND METHODS

Morphological and biochemical analyses

Morphological analysis of skeletal muscle, and biochemical assays of the individual respiratory complexes on muscle
homogenate and digitonin-treated fibroblasts were carried out as described. In fibroblasts and muscle, the specific activities were normalised to that of citrate synthase, an indicator of the number of mitochondria. In cybrids, the specific activities were not normalised, since citrate synthase is present also in mtDNA-less mitochondria which remain in the 143B ρ⁰ cells that we used as “nuclear donors”. These data are therefore presented as nanomoles of reaction product/min/mg of protein. Protein concentration was measured by the method of Lowry.

Sequence analysis

A total of 11 polymerase chain reaction (PCR) fragments encompassing the entire mtDNA were obtained from genomic DNA using pairs of 25-mer sense and antisense primers, following a standardised protocol. Each fragment was directly sequenced in both directions in an ABI 373A automated sequencer (Perkin-Elmer, USA), using the DNA sequencing kit, and the dye terminator protocol (Perkin-Elmer, USA).

Restriction fragment length polymorphism analysis

A 203 base pair fragment encompassing nucleotide positions 4258–4460 of the mtDNA Cambridge sequence was PCR amplified using a 34-mer sense oligonucleotide: 5'-ACCTAA GAAATATGTCTGATAAAAGAGGAGCT-3' (from nucleotide positions 4258-4292) and the following 30-mer antisense oligonucleotide: 5'-AAGGGTATAACCAACATTTTCGGGGTATGG-3'. The underlined SacI specific restriction site (GAGCTC) was used for restriction fragment length polymorphism (RFLP) analysis. After digestion with SacI, the cleaved 171 bp and 32 bp restriction fragments, corresponding to the mutant species, were separated from the 203 bp uncut fragment, corresponding to the wild-type species, by non-denaturing polyacrylamide-TBE gel electrophoresis. For last cycle PCR experiments, 0.2 MCl/sample of 32P-DCTP was added in the last PCR cycle. In this set of experiments, after fixation in 10% acetic acid, 25% isopropanol, the TBE gel was washed for 20 minutes in Amplify fluographic reagent (Amersham) and layered onto a phosphor imaging screen (BioRad). After overnight exposure, autoradiography was carried out in a molecular imager apparatus (BioRad). The mutation load was measured by densitometric analysis.

Fibroblast and cybrid cell cultures

Fibroblast cell lines were cultured continuously in Dulbecco’s modified Eagle’s medium with 10% FCS. Transmitochondrial cybrids were obtained by polyethylene glycol fusion, followed by selection in a uridine-free medium, as described. Cytoplasts derived from cytochalasin treated patient’s fibroblasts were fused with a ρ⁰ derivative of the human osteosarcoma 143B cell line. The absence of mtDNA in ρ⁰ cell lines, and its presence in transmitochondrial cybrids was confirmed by PCR using pairs of primers that amplify the D-loop region, as described. The presence of the appropriate nuclear genotypes in transmitochondrial cybrids was confirmed by fragment length polymorphism analysis using the highly polymorphic marker D11S533, as described.

In vivo mtDNA translation

Mitochondrial DNA encoded proteins of semiconfluent cybrid cells, from one clone of patient II-1, were labelled with 35S-methionine (43.5 TBq/mmol) (American Radiolabeled Chemicals) in the presence of emetine (0.2 mg/ml) for 60 min. Proteins were separated by Tricine SDS-PAGE, as described. The gel was then fixed for 30 min in 30% methanol, 10% acetic acid, soaked for 60 min in dimethyl sulphoxide, and for 120 min in 22% polyethylene oxide in dimethyl sulphoxide. After washing in distilled water for 60 min, the gel was dried and exposed for 3–4 days in a screen cassette. The autoradiography was visualised in a bioimaging analyser (Bas 5000, Fuji Photo Film).

Western blot analysis on two dimension blue native electrophoresis

For two dimension blue native electrophoresis, crude mitochondrial pellets were resuspended in 100 μl of 1.5 M 6-aminohexanoic acid, 50 mM Bis-Tris, pH 7.0. Twenty μl of 10% β-lauryl maltoside were added, and the samples were...
incubated for 15 min on ice. Clearing of the samples was performed by centrifugation at 12000×g for 20 min at 4°C. The supernatant was supplemented with 10 μl of 5% Serva Blue G in 1 M 6-aminoheptanoic acid and used for the first dimension. Proteins separated by second dimension blue native PAGE were electrotransferred on nylon filters, which were immunostained by using a monoclonal antibody specific to cyt c oxidase subunit I, as described.

RESULTS

Biochemical studies on muscle homogenate and cultured fibroblasts

The clinical picture of the index case pointed to a genetically determined, progressive neurological disorder associated with metabolic disturbances (severe obesity, glucose intolerance, hyperlipidaemia). This suspicion was corroborated by the presence of moderately elevated levels of lactate in the cerebrospinal fluid, and by brain magnetic resonance imaging and proton magnetic resonance spectroscopy findings, both reminiscent of lesions found in Leigh syndrome (fig 2).

To establish whether the gene mutation underlying the disease was carried by a nuclear or mitochondrial gene, we performed a complementation assay on transmitochondrial cybrids. The latter were produced by fusing cytoplasts derived from fibroblasts of the two affected sisters and their mother, mtDNA-less cybrids. Suitable markers were used to prove that no nuclear DNA belonging to the probands was present in the cybrid clones. The amount of cybrid mtDNA was proven by Southern blot and restriction fragment length polymorphism (RFLP) analysis of an allele specific PCR fragment using the diagnostic restriction endonuclease SacI confirmed the results obtained by nucleotide sequence analysis (fig 3C). RFLP analysis of last cycle radioactive PCR showed that the mutation was homoplasmic in mtDNA from skeletal muscle, fibroblasts and lymphocytes. The same homoplasmic mutation was detected in mtDNA extracted from lymphocytes and fibroblasts of the affected sister and in fibroblasts and skeletal muscle of the mother. Overexpression of the wild-type mtDNA in the mother's lymphocyte sample. We estimated this wild-type mt DNA as being approximately 3% of the total amount of mtDNA. The mutation was absent in 500 mtDNA control samples. As shown in fig 3D, the mutant C is conserved throughout eukaryotic species, including most of the metazoans, several plants, and fungi. In addition, this change can in principle produce an abnormal pairing with an opposite G (fig 3B), creating a distortion in the structure of the anticodon loop, which may interfere with the binding of the tRNA to the corresponding Ile codons. In addition to the 4290T>C change, we found sixteen homoplasmic polymorphisms of mtDNA that were already reported as non-pathogenic changes in the MITOMAP database (see www.mitomap.org for details). We also found a previously unreported homoplasmic 8818C>T polymorphism corresponding to a synonymous change from codon CTA to codon TTA, both specific to Leu(AGU) of ATPase6. Two additional, previously unreported changes were found in the 12S rRNA gene (745A>G) and in the 16S rRNA gene (3204C>T), respectively. These positions are not conserved in mammalian species (see www.ncbi.nlm.nih.gov/PMGifs/Genomes/organelles.html for details). The same changes were found in subjects II-2 and I-2.

Biochemical studies on cybrid

To establish whether the gene mutation underlying the disease was carried by a nuclear or mitochondrial gene, we performed a complementation assay on transmitochondrial cybrids. The latter were produced by fusing cytoplasts derived from fibroblasts of the two affected sisters and their mother, mtDNA-less cybrids. Suitable markers were used to prove that no nuclear DNA belonging to the probands was present in the cybrid clones. These results clearly indicated that a deleterious mutation in the probands' mtDNA was responsible for the biochemical defects in the respiratory chain and, as a consequence, of the clinical picture in the affected individuals.

Table 1 Enzymatic activities in fibroblasts

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<tr>
<th>Subjects</th>
<th>Complex I/ citrate synthase</th>
<th>Complex II/ citrate synthase</th>
<th>Complex IV/ citrate synthase</th>
<th>Citrate synthase</th>
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<tr>
<td>I-2</td>
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<td>13.5</td>
<td>56.4</td>
<td>136.7</td>
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<tr>
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<td>8.2</td>
<td>12.9</td>
<td>57.6</td>
<td>162.0</td>
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<tr>
<td>II-2</td>
<td>9.0</td>
<td>11.2</td>
<td>79.3</td>
<td>150.5</td>
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<tr>
<td>Controls (30)</td>
<td>37.8±17.7</td>
<td>14.3±3.5</td>
<td>114.0±39.3</td>
<td>147.6±27.7</td>
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</table>

Table 2 Enzymatic activities in cybrids

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<th>Complex IV (nmol/min/mg)</th>
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</thead>
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<td>I-2 (6)</td>
<td>7.3±4.1</td>
<td>52.3±22.2</td>
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<td>II-1 (2)</td>
<td>6.6±5.9</td>
<td>55.0±59.0</td>
</tr>
<tr>
<td>II-2 (5)</td>
<td>9.5±3.9</td>
<td>45.0±22.8</td>
</tr>
<tr>
<td>Controls (19)</td>
<td>26.3±11.6</td>
<td>117.9±55.5</td>
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Sequence analysis of mtDNA

Sequence analysis of the entire mtDNA molecule of the index patient (subject II-1) revealed the presence of a mutation replacing a wild-type T at nucleotide position 4290 with a C (fig 3A), in the anticodon loop of the tRNA^Ile^ cloverleaf structure (fig 3B). Restriction fragment length polymorphism (RFLP) analysis of an allele specific PCR fragment using the diagnostic restriction endonuclease SacI confirmed the results obtained by nucleotide sequence analysis (fig 3C). RFLP analysis of last cycle radioactive PCR showed that the mutation was homoplasmic in mtDNA from skeletal muscle, fibroblasts and lymphocytes. The same homoplasmic mutation was detected in mtDNA extracted from lymphocytes and fibroblasts of the affected sister and in fibroblasts and skeletal muscle of the mother. Overexpression of the wild-type mtDNA in the mother's lymphocyte sample. We estimated this wild-type mt DNA as being approximately 3% of the total amount of mtDNA. The mutation was absent in 500 mtDNA control samples. As shown in fig 3D, the mutant C is conserved throughout eukaryotic species, including most of the metazoans, several plants, and fungi. In addition, this change can in principle produce an abnormal pairing with an opposite G (fig 3B), creating a distortion in the structure of the anticodon loop, which may interfere with the binding of the tRNA to the corresponding Ile codons. In addition to the 4290T>C change, we found sixteen homoplasmic polymorphisms of mtDNA that were already reported as non-pathogenic changes in the MITOMAP database (see www.mitomap.org for details). We also found a previously unreported homoplasmic 8818C>T polymorphism corresponding to a synonymous change from codon CTA to codon TTA, both specific to Leu(AGU) of ATPase6. Two additional, previously unreported changes were found in the 12S rRNA gene (745A>G) and in the 16S rRNA gene (3204C>T), respectively. These positions are not conserved in mammalian species (see www.ncbi.nlm.nih.gov/PMGifs/Genomes/organelles.html for details). The same changes were found in subjects II-2 and I-2.

In vivo translation studies

To evaluate the consequence of the mutation on mtDNA translation, an in vivo assay was performed on a mutant cybrid clone from the index case, and control (143B.206) cells. As shown in fig 4, the mtDNA-specific translation pattern obtained from the mutant cell line was similar to that of the control cell line. However, the band set corresponding to a synonymous change from codon CTA to codon TTA, both specific to Leu(AGU) of ATPase6. Two additional, previously unreported changes were found in the 12S rRNA gene (745A>G) and in the 16S rRNA gene (3204C>T), respectively. These positions are not conserved in mammalian species (see www.ncbi.nlm.nih.gov/PMGifs/Genomes/organelles.html for details). The same changes were found in subjects II-2 and I-2.

Studies on cytochrome c oxidase assembly

To understand the consequences of the mutation on the assembly of the mtDNA-dependent respiratory chain complexes, and in particular of complex IV (cytochrome c oxidase), western blot analysis using an antibody specific to cytochrome c oxidase subunit I was carried out on equal
amounts of mitochondrial proteins, extracted from the same number of mutant cybrids and 143B.206 cells, and separated by two dimension blue native electrophoresis. Four subcomplexes (S1, S2, S3, and S4) have previously been defined by two dimension blue native electrophoresis as cytochrome c oxidase assembly intermediates of increasing molecular weight. In normal conditions, S1 corresponds to the incorporation of cytochrome c oxidase subunit I. This first step is followed by the incorporation of cytochrome c oxidase subunit IV, producing a second intermediate, S2. The third intermediate, S3, is the result of the incorporation in the nascent complex formed by cytochrome c oxidase I and IV of a large series of subunits, including cytochrome c oxidase subunits II, III, VIa, VIb, Vb, Va, VIIa, and VIIc, and VIII. Finally, fully assembled cytochrome c oxidase, corresponding to S4, is completed by the incorporation of two late subunits, cytochrome c oxidase VIa and VIIa. As shown in fig 5, in 143B.206 control cells, most of the cross reacting material specific to cytochrome c oxidase I was confined to S4, corresponding to fully assembled cytochrome c oxidase. By contrast, most of the cross reacting material in two mutant cybrid clones (from subjects II-1 and I-2) were present in intermediate subcomplexes S1-S2-S3. The fully assembled complex, S4, was reduced. The densitometrically estimated amount of assembled cytochrome c oxidase was approximately 50% in the mutants, relative to the control cell line homogenates, a percentage similar to that of the corresponding specific activities (see tables 1 and 2).

**DISCUSSION**

We found a homoplasmic mutation in the mtDNA tRNAIle [346] gene in a mitochondrial disease affecting two sisters. A third sibling, reported as having similar clinical presentation, died several years ago and could not be investigated. Surprisingly, the mutation was also present as a homoplasmic or nearly homoplasmic change in the unaffected mother, who displayed the same OXPHOS defects in both fibroblasts and cybrids as those found in the same cells from the two affected daughters.

The hypothesis that the tRNAIle mutation is pathogenic in this family is supported by the following evidence. Firstly, the rRNAIle change was the only mutation affecting a highly conserved position in the entire mtDNA sequence of the probands. Other mtDNA changes were known polymorphisms or synonymous changes that do not alter the predicted sequences of the corresponding proteins. Two additional changes affect non-conserved regions in the rRNA genes. Secondly, the mutation can alter the secondary, and possibly...
tertiary structure of the anticodon tRNA loop. Thirdly, the mutation was absent in more than 500 mtDNAs from European subjects taken as controls. Fourthly, and more importantly, the biochemical phenotype identified in fibroblast cell lines of the probands, consisting in severe, multiple defects of mtDNA-related respiratory chain complexes, was retained in all the numerous cybrid clones generated from different mutant fibroblast cell lines. This result indicates that, irrespective of the nuclear background, the OXPHOS defect segregated as a mtDNA trait in this family. Fifthly, transmission of the defect was compatible with a maternally inherited trait, since all the offspring of a mutant mother were affected. Finally, lower steady state levels of translation products corresponding to the largest mtDNA-encoded respiratory subunits were found in a cybrid clone from the index patient. This result is coherent with the hypothesis of a functional defect in a mtDNA-encoded tRNA.

The tRNAIle mutation was homoplasmic in all the available tissues of both affected siblings and in muscle and fibroblasts of their healthy mother. With the exception of a very low percentage of wild-type mtDNA detected by an ultrasensitive radioactive PCR assay in the mother's lymphocyte mtDNA, this condition is similar to that reported in most of the mtDNA mutations associated with LHON20 and MIHL.21 22 In addition, both affected members and maternal relatives from two families with hypertrophic cardiomyopathy carried the same 4300A\rightarrow G homoplasmic mutation in tRNAIle,4 and a family composed of an apparently healthy mother, a child affected by severe Leigh-like syndrome and several stillbirths or miscarriages was recently reported to carry a homoplasmic mutation in the tRNAVal gene in different tissues.4 The offspring of this family, consisting of 10 conceptions altogether, were generated by the same mother with four different partners, clearly indicating maternal inheritance of the disease trait. The mother of this family was indeed affected by a subclinical mitochondrial myopathy. However, no biochemical phenotype was detected in fibroblast cell lines or cybrids. In our case, an OXPHOS phenotype was clearly present in fibroblasts and cybrid cell lines from both mother and daughters. This result conclusively identifies mtDNA as the genome responsible for the neurological syndrome affecting the two sisters, and possibly a third one deceased long ago. By contrast, the skeletal muscle was morphologically and biochemically spared in one of the two sisters and in the mother. We do not have experimental based evidence to explain this observation, which was indeed a surprising finding since mitochondrial myopathy is common in OXPHOS disease. However, a number of mtDNA mutations have been reported to be associated with tissue specific abnormalities, in which involvement of skeletal muscle was absent. The most obvious example is provided by the LHON mutations, but also by the 8993T\rightarrow G mutation causing NARP, or by the several mtDNA mutations associated with maternally inherited deafness. The condition that is perhaps the most similar to ours is the 4300A\rightarrow G mutation in tRNAIle. This mutation, which was found as a homoplasmic change in families with maternally inherited isolated cardiomyopathy of variable penetrance, was shown to be associated with very low steady state levels of the tRNAIle transcript in heart, but not in skeletal muscle. Accordingly, a severe, combined defect of complex I and IV was detected in the heart muscle, but not in the skeletal muscle of an index case.4 These observations, including that reported in the present paper, support the concept that the phenotypes of homoplasmic mtDNA mutations are strongly influenced by still unknown, possibly multiple, factors, that not only dictate the disease penetrance.
The neurological picture was dominated by ataxia, dysarthria, and other cerebellar signs, by bilateral involvement of the optic nerve, and by several symptoms attributable to lesions of specific structures in the brainstem and mesencephalon. The lesions found by both magnetic resonance imaging and neuropathological examination were partly symmetrical, as typically seen in Leigh syndrome. However, some asymmetrical lesions were also found, such as the prominent involvement of the right cerebellar hemisphere of the index patient shown in fig 3C. The “metabolic” features, such as hyperlipidaemia and glucose intolerance, can also be attributed to the primary mitochondrial abnormality, or could be ascribed to the severe obesity affecting both patients. The nature and pathogenesis of the obesity is presently poorly understood. Delayed or impaired body growth, rather than weight gain, is a common feature in mitochondrial disease. Reduced utilisation of nutrients, endocrine abnormalities that can further decrease energy production (for example, hypothyroidism) and compulsory hyperphagia due to disturbances of the central nervous system, could in principle produce an increase of fatty mass and gross obesity. No hypothyroidism or compulsory hyperphagia were reported in our patients. This aspect of the syndrome requires further investigation.

The most striking feature of our trNA\textsuperscript{cys} mutant family was the absence of clinical and neuroradiological signs in the mother of the two probands, in spite of her showing the same biochemical defects of the patients, and being a carrier of the same mutation, that was homoplasmic in two cell types and nearly homoplasmic in a third. Another intriguing feature of our patients was the virtual absence of clinical, morphological, and biochemical abnormalities in the skeletal muscle of the probands, since myopathy is a common feature of mitochondrial disorders. Both these findings illustrate the variability in penetrance and expressivity of disease associated with homoplasmic mtDNA mutations. Compensatory effects provided by epigenetic (environmental) factors or, more likely, by different nuclear genetic backgrounds, may offer an explanation for this intriguing phenomenon. For instance, overexpression of genes such as the Er-UF elongation factor has been proven to compensate the translational defect and the consequent biochemical deficiency of specific mtDNA-trNA\textsuperscript{cys} mutations in yeast cells. The study of the differential expression profiles in affected and non-affected individuals carrying the same homoplasmic mutation of mtDNA is a promising, but yet to be set up approach to address this difficult question. We predict that homoplasmic pathogenic mutations in the mitochondrial genome represent a potentially vast, still largely overlooked and poorly understood area of mitochondrial medicine, and will stand as a new challenge in the nosological and physiopathological definition of these disorders in the next future.

FIGURE 5 Western blot analysis of cytochrome c oxidase subunit I from two dimension blue native electrophoresis performed on crude mitochondrial extracts. The upper and middle panels show the results on cybrid clones from probands I-2 and II-1; the bottom panel shows the results on 143B,206 cells taken as a control. Arrows indicate the directions of the 1st and 2nd electrophoretic run. S1–3 indicate cytochrome c oxidase assembly intermediates of increasing molecular mass. S4 corresponds to fully assembled cytochrome c oxidase.

in different individuals, but also the biochemical and clinical consequences in different tissues and, possibly, in different stages of development. Interestingly enough, the two affected sisters showed a very similar natural history of the disease. Although the younger sister (subject II-2) was more severely affected than the elder (subject I-1, index case), both developed very similar lesions, as seen by magnetic resonance imaging, and the neurological signs and symptoms were virtually identical. Both developed the same “metabolic abnormalities”, including gross obesity, glucose intolerance and hyperlipidaemia.

As frequently seen in mitochondrial encephalopathy, our patients were affected by a progressive, multisystem failure of neurological functions, particularly those localised in the brainstem and deep brain nuclei. The clinical presentation was congruent with the presence of lesions documented by magnetic resonance imaging distributed throughout the deep grey structures of the brain, similar to those found in Leigh syndrome, an early onset neurological entity attributed to several biochemical defects in the mitochondrial energy pathway. Elevated levels of lactate in the cerebrospinal fluid, an index of impaired aerobic metabolism, was accompanied by the identification of lactate accumulation in the brain lesions of the index case, as revealed by proton magnetic resonance spectroscopy. In contrast with typical Leigh syndrome, however, the onset of the disease was delayed and its course was slowly progressive in our patients.

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