Novel large-range mitochondrial DNA deletions and fatal multisystemic disorder with prominent hepatopathy

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ABSTRACT

Hepatic involvement in mitochondrial cytopathies rarely manifests in adulthood, but is a common feature in children. Multiple OXPHOS enzyme defects in children with liver involvement are often associated with dramatically reduced amounts of mtDNA. We investigated two novel large scale deletions in two infants with a multisystem disorder and prominent hepatopathy. Amount of mtDNA deletions and protein content were measured in different post-mortem tissues. The highest levels of deleted mtDNA were in liver, kidney, pancreas of both patients. Moreover, mtDNA deletions were detected in cultured skin fibroblasts in both patients and in blood of one during life. Biochemical analysis showed impairment of mainly complex I enzyme activity. Patients manifesting multisystem disorders in childhood may harbour rare mtDNA deletions in multiple tissues. For these patients, less invasive blood specimens or cultured fibroblasts can be used for molecular diagnosis. Our data further expand the array of deletions in the mitochondrial genomes in association with liver failure. Thus analysis of mtDNA should be considered in the diagnosis of childhood-onset hepatopathies.

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1. Introduction

The oxidative phosphorylation (OXPHOS) deficiencies represent one of the major causes of metabolic disorders with a prevalence estimated at 1/8500 birth [1]. These disorders represent a heterogeneous group of genetic diseases, mainly affecting brain, heart and muscle, and can be caused by genetic defects in mitochondrial (mt) or nuclear (n) DNA. OXPHOS catalyzes the oxidation of fuel molecules by oxygen and the concomitant energy transduction into ATP via five complexes, embedded in the inner mitochondrial membrane. The mitochondrial OXPHOS is made up of about 100 different proteins, 13 of which are encoded by mtDNA and the others by nDNA [2].

The high dependence of hepatocytes upon ATP production for the correct biosynthetic and detoxifying pathways explains in part why liver involvement is also a common feature in childhood OXPHOS-related diseases, particularly in the neonatal period, where it may present as neonatal acute liver failure, hepatic steatohepatitis or cirrhosis with chronic liver failure of insidious onset [3]. It is becoming obvious that conditions with markedly reduced levels of mtDNA (mtDNA depletion, MDS) frequently cause liver failure in early childhood [4], and syndromes associated with prominent hepatic disease and related to mtDNA deletions are rare [5]. Importantly, liver complications are often a late feature of a multi-system disorder, such as Leigh syndrome [6].

We herein report on the clinical, biochemical and molecular features in two children in which a multisystem disorder, resembling Pearson syndrome, was associated to a prominent hepatopathy and related to novel mtDNA large-scale rearrangements encompassing multiple genes of NADH-ubiquinone oxidoreductase (complex I).

2. Materials and methods

2.1. Case reports

Details of the clinical features of both patients are summarized in Case reports of Supplementary methods. Both patients manifested

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prominent signs of progressive hepatic failure that stated within the first 2 years of life associated with some features resembling Pearson syndrome such as pancytopenia with non-hemolitic megaloblastic anaemia, signs of pancreatic failure and severe lactic acidosis.

2.2. Tissues

Muscle and skin biopsies were obtained pre-mortem, whereas all other tissues were sampled 1 h after death once obtained informed parental consent. All tissues were immediately frozen in liquid nitrogen and stored at −80 °C until assayed. Autoptic tissues, whereas available, were obtained from age-matched controls (kids who died for no metabolic causes) and frozen following similar procedures than patients. Human fibroblasts were grown in DMEM medium supplemented with 10% foetal bovine serum, 4.5 g/L glucose and 50 µg/mL uridine.

2.3. Histological and electron microscopy studies

Frozen sections of muscle biopsy were stained for haematoxylin and eosin (HE), Gomori trichrome, cytochrome c oxidase, and succinate dehydrogenase [7]. The liver specimens were processed for light and electron microscopy. Light microscopy studies included routine stains, HE and Masson trichrome. Ultrathin sections were analysed using a Zeiss 109 electron microscope [8].

2.4. Biochemical and molecular studies

Spectrophotometric determination of respiratory chain enzymes activities in bioptic and autoptic muscle, and in autoptic liver tissue, Southern blotting, SDS–PAGE/Western blotting, genomic DNA purification and PCR amplification (in fibroblasts and autoptic tissues) used previously reported methodologies [9–11]. To define precisely the breakpoints of the deletions, we used a "primer shifting" PCR-based strategy as reported elsewhere [12], and mtDNA (GenBank ID: NC_012920) oligonucleotide primer sets 16,470–8330, 6900–16,470 and 7250–16,470 for patient 1, and sets 10,500–16,569 and 11,500–16,430 for patient 2. Fine mapping of the deletions was performed by direct sequencing using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) on an ABI3130xl automatic DNA Analyzer. Determination of the relative abundance of wild-type and deleted genomes used either an ad hoc designed "3-primer last hot-cycle" PCR method [13,14] or a quantitative real-time PCR (qPCR) assay [15,16], or both (see Supplementary methods). For SDS–PAGE, 50 µg of proteins from different tissues were loaded in a 12% denaturating gel. Immunoreactivity of the following proteins was ascertained using monoclonal antibodies purchased from Mitosciences (Eugene, OR, USA): complex I—15 kDa subunit (NDUFB4), 20 kDa subunit (NDUFB8), 30 kDa subunit (NDUFS3), and 39 kDa subunit (NDUFA9); complex II—70 kDa subunit (SDH70); complex III—core 2 subunit (core2); complex IV—subunit II (CIV-II); and complex V—subunit alfa (CVα). Reactive bands were detected using the Immobilon Western Chemiluminescent HRP Substrate Detection Kit (Millipore Corporation, Billerica, MA, USA). Fluorescence was quantified using the Quantity One Software (BioRad, Hercules, CA, USA). Each sample was run in triplicate and normalized values were averaged and compared to normal control tissues.

3. Results

In patient 1, liver histology showed a micronodular cirrhosis (Fig. 1A top) with ductular reaction and marked cholestasis. Hepatocytes were ballooned, occasionally with multinuclei, microvesicular steatosis and haemosiderosis (Fig. 1B top). At the electron

microscopy (Fig. 1C top) the liver showed an accumulation of mitochondria, which also appeared round and with few and dysregulated cristae. The liver histology in patient 2, showed preserved architecture with mild portal fibrosis (Fig. 1A bottom). Hepatocytes had an oncocytic appearance due to an increased number of mitochondria, sometimes with megamitochondria (Fig. 1B bottom). In addition, ballooning degeneration, micro and macrovesicular steatosis, cholestasis, and ductular proliferation were present. At the electron microscopy (Fig. 1C bottom) the liver showed an accumulation of mitochondria even more pronounced than in patient 1; in some mitochondria the cristae were almost absent. Moreover, an accumulation of lipids drops were observed.

Spectrophotometric determination of the activities of respiratory chain complexes in patient 1 showed 73% reduction of complex I in autoptic muscle homogenates (the muscle biopsy specimen was inconsistent for spectrophotometric studies), and multiple defects in autoptic liver with undetectable activity of complex I, 12% of residual activity of complex III and 39% residual activity of complex IV, upon correction for the levels of citrate synthase. In patient 2, we detected an isolated defect of complex I (residual activity 40%) in bioptic and autoptic muscle, as well as in autoptic liver, with 17% residual activities compared to the lowest range of controls. In mitochondria isolated from cultured fibroblasts complex I activity was almost undetectable in patient 1, and 50% reduced in patient 2.

In fibroblasts Southern blot analysis identified, in addition to the wild type molecules, two smaller bands sized approximately 11.0 Kb in patient 1 (Fig. 2A) and 13.0 Kb in patient 2 (Fig. 2B). The bands corresponded to heteroplasmic mtDNA deletions. There was no evidence for reduced amount of mitochondrial genomes (data not shown). Fine mapping of the deletions disclosed that patient 1 harboured a 5312 base pairs (bp) long deletion, extending from nucleotide nt. 10,090 in NADH-dehydrogenase, subunit 3 (ND3) to nt. 15,402 in cytochrome b (CYTB), and patient 2 a 2837-bp, from nt. 12,366 in NADH-dehydrogenase, subunit 5 (ND5) to nt. 15,203 in CYTB. The novel deletion identified in patient 1 removed five tRNAs (R-H-S2-L2- and E), large part of complex I ND subunits (part of ND3, ND4L, ND4, ND5, and ND6), and a small region of cytochrome b; whereas, the new deletion in patient 2 removed the sequences of ND5, ND6, part of cytochrome b and the tRNAGlu gene. The deletions were not flanked by direct repeats.

Using radiolabelled 3′-PCR, the very high levels of deleted mtDNA was found in liver tissue of both patients (92% and 89%, respectively in patients 1 and 2); whereas the other tissues showed values ranging from 68% to 86% in patient 1, and from 33% to 94% for patient 2 (Supplementary Fig. 1) with very high levels particularly in kidney and pancreas. Similar data were observed using qRT-PCR method (Supplementary Fig. 2). Values obtained from both methods are compared in Table 1.

Western blot analysis showed a clear decreased amount of NDUFB4 and NDUFB8 complex I nuclear encoded subunits in tissues from patient 2 (Fig. 3A and B), while other nuclear encoded subunits such as NDUFA9, NDUFS3 were normally present or only mildly reduced. In detail, NDUFB4 and NDUFB8 subunits were not detected in liver, markedly reduced in kidney and relatively reduced in skeletal muscle, but seemed to be preserved in heart. Complex II, III, IV and V subunits were normally expressed (Fig. 3C). No sufficient material was available for patient 1.

4. Discussion

Single deletions of mtDNA are usually associated with 3 major clinical conditions: Kearns–Sayre syndrome (KSS) (MIM 530000), Pearson syndrome (MIM 557000), and chronic progressive external ophthalmoplegia (CPEO) (MIM 157640). Typically, single mtDNA deletions are sporadic events occurring in isolated members of a
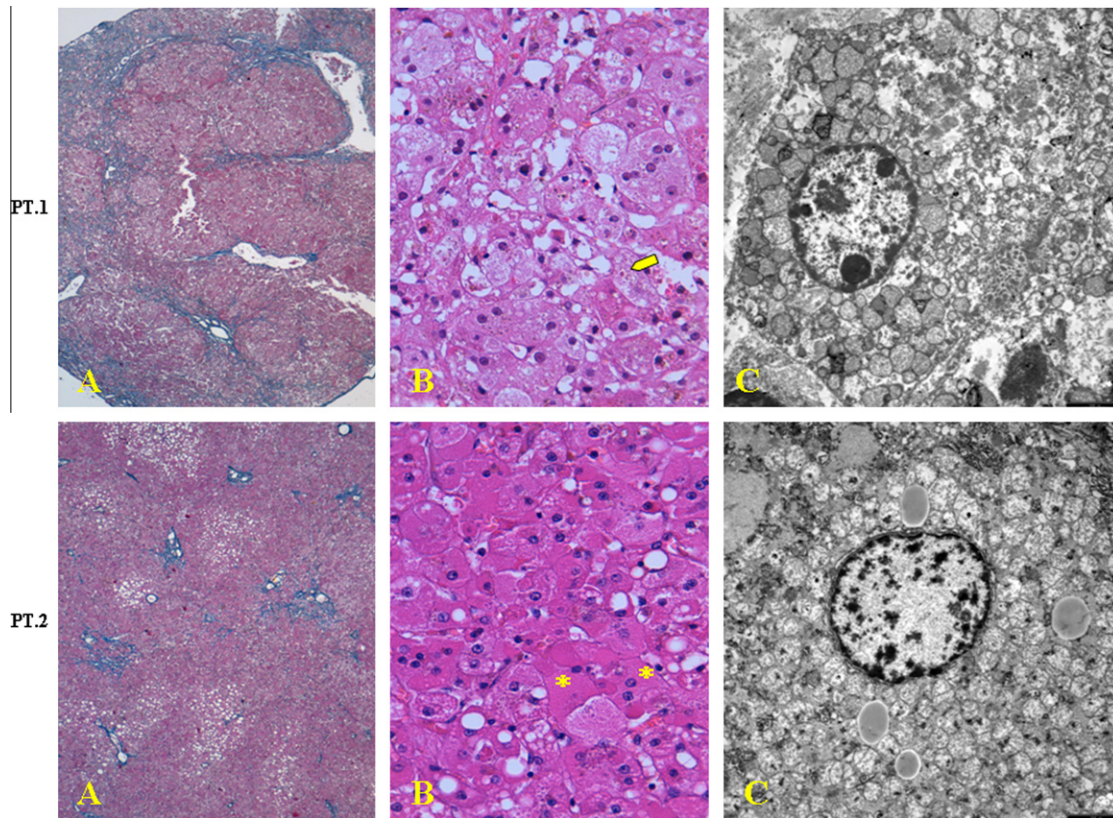
Fig. 1. Histological and electron microscopy in liver tissue of patient 1 (top) and patient 2 (bottom). (A) Masson trichrome, 4×; (B) hematossilin eosin (HE), 40×; (C) electron microscopy (EM), 1500 magnification (Bars, 2500 nm). The Masson thrichrome showed in patient 1 (top-A) disturbed architecture due to fibrotic septa around hepatic nodules. The HE (top-B) evidences ballooned hepatocytes with multinuclei and microvesicular steatosis. Sometimes megamitochondria are present (arrow). With EM (top-C) the mitochondria appeared round with a less electron-dense matrix and markedly reduced cristae. In patient 2 by Masson trichrome (bottom-A) the architecture is preserved; only mild portal fibrosis is evident. The HE (bottom-B) shows oncocytic hepatocytes rich in mitochondria (asterisks). The EM (bottom-C) displays a heavy accumulation of mitochondria throughout the hepatocytes. Again, note the loss of cristae in these mitochondria. Lipids drops are present.
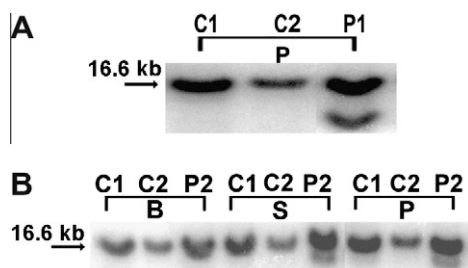
Fig. 2. Southern blotting of total fibroblasts DNA from patient 1 (A) and patient 2 (B). DNA digestion of patient 1 was performed using *Pvu*II (P) restriction enzyme; for patient 2: *Pvu*II (P), *Bam*HI (B), and *Sna*BI (S) were used. Total human mtDNA was used as a probe. The bands at 16.6 kb represent the wild-type mtDNA while the smaller ones represent partial deleted mtDNAs. P1 and P2: patient 1 and 2, respectively. C1 and C2: controls 1 and 2, respectively.

Table 1
Relative percentage of mtDNA deleted molecules in post-mortem tissues and in blood from patients 1 and 2 by using two different quantitative techniques.

	Last cycle hot-PCR	qRT-PCR
Patient 1		
Fibroblasts	67.8 ± 3.1	54.6 ± 4.6
Liver	91.6 ± 9.6	89.3 ± 1.1
Spleen	86.2 ± 0.1	82.1 ± 6.3
Skeletal muscle	83.8 ± 2.7	80.8 ± 1.7
Pancreas	84.6 ± 3.1	82.1 ± 1.2
Kidney	83.2 ± 2.3	89.5 ± 0.4
Patient 2		
Fibroblasts	33.6 ± 3.5	33.9 ± 10.7
Heart	66.8 ± 2.2	62.2 ± 3.8
Liver	88.7 ± 3.0	93.5 ± 0.7
Skeletal Muscle	79.2 ± 3.7	80.5 ± 1.4
Pancreas	83.5 ± 3.3	92.3 ± 0.4
Lung	86.6 ± 2.8	83.6 ± 0.9
Kidney	89.0 ± 2.6	94.2 ± 0.3
Blood	83.3 ± 0.1	n.d.
Adrenal Gland	89.8 ± 2.4	86.1 ± 5.4

n.d.: not-determined.

family, but, exceptionally, they can also be transmitted through the germline [17].

The patients herein described both present with a multisystem disorder mainly affecting the liver with signs of Pearson's syndrome and associated to a single, large-scale mtDNA deletion. The deletions found in our patients have never been described before, and were ubiquitously distributed in all tissues. They removed at least one tRNA and several subunits of complex I, which is consistent with the profound biochemical defect identified in all examined tissues. The expected profound defect in protein content was demonstrated by Western blotting analyses with a prominent

reduction of complex I subunits. Unexpectedly we found an uneven reduction of some complex I nuclear encoded subunits in some tissues compared to others. NDUFB4 and NDUFB8 subunits were markedly reduced only in liver, kidney and skeletal muscle while NDUFA9, NDUFS3 subunits appeared to be comparatively normal or only mildly reduced. While it is complex to explain the cause of uneven distribution of complex I subunits in different
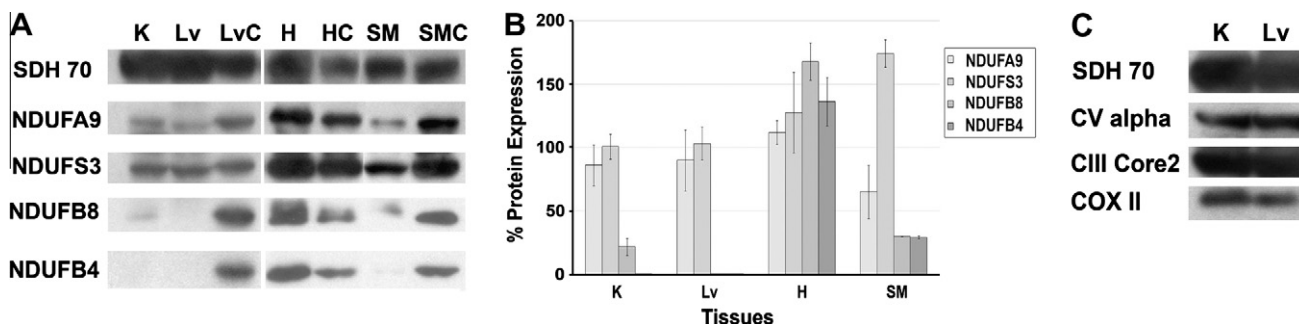
Fig. 3. Western blotting in post-mortem tissues of patient 2. (A) Homogenates from post-mortem tissues were separated by SDS–PAGE, and antibodies against subunits of complex I (NDUFA9, NDUFS3, NDUFB8, NDUFB4), and complex II (SDH 70 kDa) were used; (B) Histograms corresponding to the normalized expression protein. K, Lv, H, SM: kidney, liver, heart and skeletal muscle from patient; LvC, HC, SMC: liver, heart and skeletal muscle from control; (C) SDS–PAGE of patient's kidney and liver using antibodies against complex II (SDH 70 kDa), complex V (alpha), complex III (core 2), and complex IV (COX II). Complex I subunits content was normalized to the mitochondrial protein SDH70 used as a control for equal loading. All tissues were referred to its own control, except for kidney (control was not available) that was normalized to liver control, considering the similar amount of protein subunits seen in (C). Low content of NDUFB8 and NDUFB4 subunits is evident in liver, kidney, and skeletal muscle, whereas NDUFA9, NDUFS3 as well as complex II, III, and IV subunits are normally expressed.

tissues we have some arguments to explain the unequal reduction of complex I nuclear encoded NDUFB4 and NDUFB8 subunits compared to others analysed, i.e. NDUFA9, NDUFS3. Complex I is the largest and least understood component of the respiratory chain. It consists of 45 different subunits that assemble together into a structure of ∼1 MDa [18]. The assembly of subunits into complex I has proved to be a very puzzling problem to address, complicated by the size of the enzyme and its dual genomic control. Newly imported subunits, encoded by the nuclear genome must assemble in coordination with subunits encoded by mtDNA. Loss of many of the mtDNA-encoded ND subunits and underexpression of some of those coded by nDNA likely make the holocomplex less stabile and partially assembled [19]. For instance, the ND1, ND4, and ND6 subunits are essential for correct assembly of complex I, since there is evidence that their mutations are associated with markedly reduced levels of the enzyme [20–22]. Therefore, it is reasonable to think that in our patients the lack of many ND subunits make the holocomplex less stabile and partially assembled. Moreover, it has been seen that in the absence of mtDNA-encoded subunits, the levels of some nDNA-encoded subunits of the peripheral arm remain unchanged [22] as confirmed by our Western blotting experiments. In fact, our results display a normal or mildly reduced content of NDUFA9 and NDUFS3 subunits, which are both components of the peripheral arm [23–26]. On the other hands, the preferential loss in our experiments of the two smaller nuclear encoded subunits NDUFB4 and NDUFB8 could be explained by their position, being part of the subcomplex Iβ, which is composed mainly by the mtDNA subunits and form the hydrophobic membrane arm of complex I [27].

In agreement with other studies, we observed a wide tissue distribution of deleted genomes [28] and it is likely that the functional status of each tissue will ultimately depend upon the accumulation of deleted mtDNA species [29]. Our results suggest a gross correlation between the amount of mtDNA deleted molecules and the content of related protein. In fact, liver, kidney and skeletal muscle showed the highest level of deletion and the lower content of NDUFB4 and NDUFB8 subunits. Whereas in heart, in which the amount of deletion is below 70%, we reveal a normal amount of complex I subunits. These findings lead us to hypothesise a possible threshold effect when the mtDNA deleted molecules exceed the 70%.

Hepatic involvement in primary (i.e. genetic) mitochondrial disorders rarely manifests in adulthood, but is a common feature in childhood respiratory chain disease, particularly in the neonatal period [30,31]. In some infants, liver dysfunction may spontaneously reverse or remain stable, but in some there is rapid progression to cholestasis, coagulopathy, and ascites. Biochemical studies reveal single or multiple respiratory chain enzymes defects, which

may be restricted to the liver and remain undetectable in skeletal muscle or skin fibroblasts [6]. Prominent liver involvement is rarely related to primary pathogenic point mutations [32], or single deletions of mtDNA [33], but is most often associated with conditions characterized by reduced amounts of mtDNA in affected tissues and grouped as MDS [34–36].

It is well established that single mtDNA deletions are generally associated to Pearson's [37], KSS [38], CPEO syndromes [39] and that surviving patients with Pearson can later present with a KSS [40]. More rarely other syndromes have been reported to be associated to mtDNA rearrangements such as the *Villous Atrophy syndrome* [41], and the "common" mtDNA deletion once associated to early onset *liver and renal failure* [33]. Moreover, in addition to KSS and CPEO syndromes, the mtDNA deletions have been detected also in patients with chronic tubulointerstitial nephropathy [42,43].

When suspecting a mitochondrial disorder with prominent hepatic involvement the biochemical defects may not always be apparent in unaffected tissues, and a liver biopsy may be indicated [6]. However, our experience shows that mtDNA deletions can be easily detected in patients' cultured fibroblasts as well as in blood cells. This confirms previous experience [28] suggesting less invasive techniques, particularly in neonates, such as cultured skin fibroblasts or blood sample that can be tested preliminarily for mtDNA deletions before analysing nuclear genes related to mtDNA depletion.

Regardless of the ultimate pathomechanisms, our data further expand the array of deletions in the mitochondrial genomes and its association with liver failure, and as such analysis of mtDNA should be considered in the diagnostic algorithm of childhood-onset hepatopathies.

Finally, we used two different methods to measure the amount of mtDNA-deleted molecules, the radiolabelled 3′-PCR and the qRT-PCR. The results obtained allow us to suggest the use of the faster and safer qRT-PCR instead of the radiolabelled 3′-PCR method.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.10.049.

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