Research Article

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Severe respiratory complex III defect prevents liver adaptation to prolonged fasting

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Background & Aims: Next generation sequencing approaches have tremendously improved the diagnosis of rare genetic diseases. It may however be faced with difficult clinical interpretation of variants. Inherited enzymatic diseases provide an invaluable possibility to evaluate the function of the defective enzyme in human cell biology. This is the case for respiratory complex III, which has 11 structural subunits and requires several assembly factors. An important role of complex III in liver function is suggested by its frequent impairment in human cases of genetic complex III defects.

Methods: We report the case of a child with complex III defect and acute liver dysfunction with lactic acidosis, hypoglycemia, and hyperammonemia. Mitochondrial activities were assessed in liver and fibroblasts using spectrophotometric assays. Genetic analysis was done by exome followed by Sanger sequencing. Functional complementation of defective fibroblasts was performed using lentiviral transduction followed by enzymatic analyses and expression assays.

Results: Homozygous, truncating, mutations in *LYRM7* and *MTO1*, two genes encoding essential mitochondrial proteins were found. Functional complementation of the complex III defect in fibroblasts demonstrated the causal role of *LYRM7* mutations. Comparison of the patient's clinical history to previously reported patients with complex III defect due to nuclear DNA mutations, some actually followed by us, showed striking similarities allowing us to propose common pathophysiology.

Conclusions: Profound complex III defect in liver does not induce actual liver failure but impedes liver adaptation to prolonged fasting leading to severe lactic acidosis, hypoglycemia, and hyperammonemia, potentially leading to irreversible brain damage.

Lay summary: The diagnosis of rare genetic disease has been tremendously accelerated by the development of high throughput sequencing technology. In this paper we report the investigations that have led to identify *LYRM7* mutations causing severe hepatic defect of respiratory complex III. Based on the comparison of the patient's phenotype with other cases of complex III defect, we propose that profound complex III defect in liver does not induce actual liver failure but impedes liver adaptation to prolonged fasting.

Introduction

Classical mitochondrial diseases result from defects of the mitochondrial oxidative phosphorylation pathway (OXPHOS). They present with diverse clinical features [1]. Liver failure is frequently reported in severe pediatric cases [2]. Its mitochondrial origin is often diagnosed by the presence of defective OXPHOS activities in liver. However, because of the possibility of improper sample preservation or nature, the diagnosis cannot solely rely on these defects but requires identification of the genetic cause of the disease. That identification has recently been tremendously facilitated by next generation sequencing.

Genetic causes of mitochondrial liver failure may be classified with respect to the type of OXPHOS defect. The most frequent are combined defects involving OXPHOS complexes with mitochondrial DNA (mtDNA)-encoded subunits, caused often by mtDNA depletion (profound decrease of the mtDNA amount) [3–6] or defects of the mtDNA translation [7–9]. In this group, liver

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Abbreviations: OXPHOS, oxidative phosphorylation pathway; mtDNA, mitochondrial DNA.

functions are globally altered associating synthesis failure with coagulopathy, cytolysis, and cholestasis often progressing towards cirrhosis. These diseases are often multisystem disorders, associating neurological deficits to the liver failure.

Much rarer cases of liver failure have been associated with an isolated OXPHOS defect, which is most often respiratory complex III (ubiquinol cytochrome c oxidoreductase, EC 1.10.2.2). In these cases the liver alterations seemed to differ according to the causal gene. Global liver failure, resembling that observed with multiple OXPHOS defects, has been described in severe, early onset, diseases due to mutations of *BCS1L* gene encoding a complex III assembly factor [10,11], acute episodes of hypoglycemia and hyperammonemia have been associated with mutations of *UQCRB*, *CYC1* and *UQCRC2* genes encoding complex III structural subunits [12–14], and normal liver functions have been reported in diseases due to mutations of genes encoding either complex III assembly factors (*TTC19* [15], *UQCC2* [16], *UQCC3* [17] and *LYRM7* [18,19]) or structural subunits (*MT-CYB* [20], *UQCRQ* [21]). The role of complex III in liver function thus appears disputable.

We here report a patient with liver complex III defect. Although exome sequencing revealed two genes, *MTO1* and *LYRM7*, both carrying homozygous truncating mutations and both encoding essential mitochondrial proteins, functional complementation by lentiviral vectors demonstrated that the *LYRM7* mutations were causing the complex III defect. Comparison of the patient's clinical history to previously reported patients with complex III defect, some actually followed by us, led us to propose common pathophysiology for several complex III defects whereby profound complex III defect in liver does not induce actual liver failure but impedes liver adaptation to prolonged fasting. Lack of appropriate metabolic treatment of the fasting-induced lactic acidosis, hypoglycemia, and hyperammonemia may result in irreversible brain damage.

Materials and methods

Patient

The patient was the third child of first cousins parents. She first presented at 6 months of age with ketoacidosis, hyperglycemia and glycosuria. Intravenous injection of insulin induced hypoglycemia without correcting ketonuria. The child was transferred to La Rabta hospital in Tunis. At her arrival she was hypotonic, polypneic and presented with mild hepatomegaly. Biological tests revealed compensated metabolic acidosis (pH 7.42, bicarbonate 15.7 mM, pCO_2 24 mmHg), hyperlactatemia (5.2 mM), and hyperlactatorrachia (8 mM) with mild hyperammonemia (64 μM, N <50), but normal liver enzymes and coagulation factors. Very high lactate, 3 OH butyrate and 2-ketoglutarate were found on urinary organic acids profiling. Electrocardiogram, echocardiography, kidney functions, and electroencephalogram were normal.

The further disease course was marked by episodes of metabolic crises associating metabolic acidosis with hyperlactatemia, ketonuria and hypoglycemia; ammonia was not measured. A severe episode, lasting three days and associated with coma, occurred at one year of age. It was followed by irreversible neurological damage with loss of contact, profound axial hypotonia, peripheral hypertonia, horizontal nystagmus, and deglutition troubles. Hepatic and skin biopsies were performed at that period. The child died shortly afterwards during a recurrent metabolic crisis at 20 months of age.

Samples

All analyses were performed after written informed consent from the parents of the child according to the local institution rules. A needle liver biopsy was immediately frozen in liquid nitrogen, stored at −80 °C until enzymatic assays. Primary fibroblasts were derived from a forearm skin biopsy as described in [22].

DNA samples were obtained from liver and fibroblasts using standard procedure with sodium dodecyl sulfate (SDS) and proteinase K digestion followed by phenol extraction and isopropanol precipitation. DNA samples from blood were obtained using QiaAmp DNA minikit (Qiagen, Courtaboeuf, France). RNA samples from fibroblasts were obtained using the miRNeasy kit (Qiagen, Courtaboeuf, France).

Mitochondrial fractions were prepared from 2 T175 fibroblasts flasks according to [23].

Mitochondrial activities

Spectrophotometric assays for the respiratory complexes I, II, III and IV, as well as citrate synthase activities were performed according to [24].

Western blot analysis

Proteins from mitochondrial pellets were separated by blue native polyacrylamide gel electrophoresis (PAGE) [25] or by SDS-PAGE on a linear 4 to 20% gradient polyacrylamide gel (Biorad, Hertfordshire, UK). Antibodies against UQCRC2 and UQCRFS1 were kindly given by Dr Catherine Godinot, France [26], antibodies against MT-ND1 and against MT-CO2 were produced in our group [27]; the other antibodies were commercially available: anti-SDHA, anti-ATP5B and anti-LYRM7 were from Abcam (France), anti-MTO1 from Proteintech (USA) and anti-ACTB from Sigma-Aldrich (USA). They were visualized using peroxidase-conjugated secondary antibodies (Sigma-Aldrich, USA) and Pierce™ enhanced chemiluminescence (ECL) Western blotting substrate (Life Technologies, USA). Western blot signals were recorded on a Fusion ultra-sensitive camera platform (Vilber Lourmat, Germany) and quantified using the Fusion attached software. Results were normalized to the control run on the same gel and to complex II, either the whole complex II after blue native gel electrophoresis or SDHA subunit after SDS gel electrophoresis.

Exome sequencing

Exome sequencing and variant filtering was essentially performed as described previously [28]. In brief, coding DNA fragments were enriched with the SureSelect Human All Exon 50 Mb V4 Kit (Agilent, Santa Clara, CA, USA) and sequencing was performed on a HiSeq2000 system (Illumina, San Diego, CA, USA). Reads were aligned to the human genome assembly hg19 (UCSC Genome Browser) with Burrows-Wheeler Aligner (version 0.7. 5) and detection of genetic variation was performed using SAMtools (version 0.1.19), PINDEL (version 0.2.5a7), and ExomeDepth (version 1.0.0). 96.9% of the target was covered at least 20-fold.

Lentiviral gene transfer

LYRM7 cDNA was purchased from DNASU Plasmid Repository (Clone ID HsCD00514704) and cloned into the pLenti6.3/V5-TOPO® vector system (Invitrogen, Life technologies) for subsequent lentiviral-mediated expression in skin fibroblast cell lines using the ViraPower HiPerform Lentiviral TOPO Expression Kit (Invitrogen, Life technologies) [29]. The corresponding *MTO1* clone was already available [30]. We also constructed a third lentivirus for expression of the *MTO1* cDNA sequence but with Geneticin resistance [31]. Stably transduced fibroblasts were selected using blasticidin (Invitrogen) and/or Geneticin (Sigma-Aldrich).

RNA analyses

Reverse transcription of 2 micrograms total RNA was performed with the Transcriptor First strand cDNA synthesis kit (Roche Diagnostics, USA). The cDNA steady-state was quantified using LightCycler 480 SybrGreen master mix (Roche Diagnostics, USA). Serial dilutions of linearized pGEM-T plasmids with the amplification products as insert, allowed absolute quantification of the cDNA in copy numbers per μl. Results were expressed as copy numbers per 1000 *ACTB* copies.

Statistical methods

Statistics were performed with SigmaPlot 12.5 software. Non-parametric tests were used unless the data were shown to have a normal distribution allowing the use of parametric tests. The numbers given indicate independent measurements. Mean comparisons were performed with Mann-Whitney *U* test or