Children's toxicology from bench to bed - Liver Injury (4): Mitochondrial respiratory chain disorder and liver disease in children

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ABSTRACT — Objectives: The present study was aimed to ascertain the contributions of mitochondrial respiratory chain (MRC) enzymes to the development of liver failure and to the liver pathophysiology of metabolic liver diseases. Methods: We investigated liver samples obtained from 8 patients with liver failure due to unknown etiology and from 15 patients with metabolic disease: ornithine transcarbamylase deficiency, 6 cases; Wilson disease, 3 cases; methylmalonic aciduria (MMA); 3 cases, neonatal hemochromatosis, 2 cases. The estimation of MRC enzymes was carried out by the following independent methods; i) blue native polyacrylamide gel electrophoresis (BN-PAGE) in gel enzyme staining, ii) BN-PAGE western blotting, iii) *in vitro* MRC enzyme assay. Furthermore, we estimated the quantities of mtDNA and nDNA using qPCR. Result: 4 cases with liver failure showed low activities and protein levels of complex I, III and IV. We also performed qPCR and estimated the ratio mtDNA/nDNA using these samples. They all exhibited extremely low ratio. They were diagnosed as mtDNA depletion syndrome. All cases except MMA cases exhibited mildly or moderately suppressed activities of complex I-IV. However, the respective protein levels remained almost normal. MMA cases exhibited low activities and protein levels of complex I, III and IV. In particular, their low activities and protein levels of complex I were noticeable. They all exhibited normal ratios of mtDNA to nDNA. Conclusion: MRC defect might be an etiology of liver failure in a considerable number of patients in Japan. The present study suggested that considerable disturbance of MRC occurs in children with metabolic diseases and possibly modifies the pathophysiology.

Key words: Mitochondrial respiratory chain disorder, Mitochondrial DNA depletion syndrome, Metabolic liver disease, Methylmalonic aciduria

INTRODUCTION

Congenital disorders of the mitochondrial respiratory chain enzymes are the most common group of inborn errors of metabolism, affecting at least 1 in 5,000 individuals (Skladal *et al.*, 2003; Thorburn, 2004; Munnich and Rustin, 2001). It can affect most organ systems, alone or in combination, so it is not surprising that these disorders could be associated with liver disease presenting as liver failure.

A considerable number of patients with liver failure remain undiagnosed in Japan. They often show liver steatosis, suggesting the presence of mitochondrial damage. On the other hand, mitochondrial damage secondary to metabolic disease, which should modify the liver patho-

physiology, has never been investigated sufficiently.

We present here mitochondrial respiratory disorder and liver disease, especially mitochondrial DNA depletion syndrome and secondary effect to metabolic liver disease.

MATERIALS AND METHODS

Subject

We investigated the mitochondrial respiratory chain enzymes in liver samples obtained from 8 patients with liver failure due to unknown etiology (Table 1) and from 15 patients with metabolic disease: ornithine transcarbamylase deficiency 6 cases; Wilson disease 3 cases; methylmalonic aciduria (MMA) 3 cases, neonatal hemochromatosis 2 cases (Table 2).

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Table 1. Case profiles with liver failure unknown etiology

	Case 1	Case 2	Case 3	Case 4
Onset age	Day 8	Day 2	8 months	4 months
Family history	Elder brother (+)	Elder sister (+)	Elder brother (+)	Younger sister (+)
Symptoms	Low birth weight Feeding difficulty Nystagmus MR	Tachypnea, Hypoglycemia MR	Failure to thrive MR Intractable diarrhea	Failure to thrive Feeding difficulty Hypotonia Deafness
Liver function				
GOT / GPT (IU/l)	120 / 46	49 / 215	109 / 42	390 / 218
T-Bil / D-Bil (mg/dl)	2.5 / 1.7	9.6 / 1.4	0.7 / -	1.6 / -
ChE (U/l)	179	-	-	73
PT (%)	16	2-4 (INR)	63	35
Lactate / pyruvate (mmol/l)	2.9 / 0.13	20.9 / 0.27	1.46 / 0.07	1.76 / 0.1
Others	AFP 3.4 x 10 ⁵ ng/ml			
Pathology	Micro / Macrovesicular steatosis, Fibrosis (mild - moderate) in portal area (P-P bridging)			
Transplantation	8 months	Not performed	Scheduled	Not performed
Status	Alive	Dead	Alive	Dead

	Case 5	Case 6	Case 7	Case 8
Onset age	5 months	2 months	4 months	1 months
Family history	Not particular	Not particular	Not particular	Not particular
Symptoms	Failure to thrive Feeding difficulty	Failure to thrive Hypoglycemia Hypotonia MR	Heart Failure (EFE) Hypotonia	Failure to thrive Jaundice
Liver function				
GOT / GPT (IU/l)	489 / 176	209 / 92	unknown	328 / 129
T-Bil / D-Bil (mg/dl)	5.7 / 4.3	3.7 / 2.2		35.4 / 25.6
ChE (U/l)	105	198		150
PT (%)	60	33.2		22
Lactate / pyruvate (mmol/l)	3.2 / 0.1	1.6 / 0.1	63.3 / -	unknown
Others				AFP > 5.0 x 10 ⁴ ng/ml
Pathology	Macrovesicular steatosis	Nodular cirrhosis Extensive fibrosis	Macrovesicular steatosis	Extensive fibrosis
Transplantation	Scheduled → Stopped	9 months	Not performed	Not performed
Status	Dead	Alive	Dead	Dead

Determinations of enzyme activities

Activities of respiratory chain complexes I, II, III and IV were assayed for the crude post-600 g supernatant of the liver samples as described previously (Rahman *et*

al., 1996; Murayama *et al.*, 2008). Each complex activity was presented as a percent ratio relative to the mean value obtained from 35 healthy controls. Together, for each patient, the percent ratios of complex I, II, III and IV

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activities to the citrate synthase (CS) as a mitochondrial enzyme marker or complex II activity were calculated.

BN-PAGE western blotting

Expressions of the mitochondrial respiratory chain complex I, II, III and IV proteins in the liver were examined by western blotting using blue native polyacrylamide gel electrophoresis (BN-PAGE) according to the methods described previously (Dabbeni-Sala *et al.*, 2001; Schägger *et al.*, 1988). Ten µg weight of the protein in mitochondria-enriched fraction was separated by BN-PAGE. Immunostaining was performed using a monoclonal antibody specific for the 39 kD subunit of complex I, 70 kD subunit of complex II, core 1 subunit of complex III and subunit 1 of complex IV (Molecular Probes, Eugene, OR, USA).

Quantitative PCR

The quantitative estimation of mtDNA was performed by the real-time amplification of fragments of nicotinamide adenine dinucleotide dehydrogenase 1 (ND1) in mtDNA genome, as previously described (Pagnamenta *et al.*, 2006; He *et al.*, 2002). To determine the overall abundance of mtDNA, we compared the real-time amplification of ND1 with single-copy nuclear reference gene (exon 24 of the cystic fibrosis transmembrane conductance regulator (CFTR) gene; chosen on account of the lack of single-nucleotide polymorphisms). For both experiments, DNA from six adult liver samples (from needle biopsies, obtained with informed consent) was used as controls,

and results are the means of four independent runs, with samples assayed in triplicate in each run.

RESULTS AND DISCUSSION

8 cases with liver failure showed extremely low activities and protein levels of complex I, III and IV (Figs. 1 and 2). We also performed qPCR and estimated the

Table 2. The cases with metabolic liver disease which were investigated respiratory chain enzymes

6 cases of OTC Deficiency	
Male	3 cases
Female	3 cases
4 cases of Wilson Disease	
Wilsonian fluminant	3 cases
Liver cirrhosis	1 case
2 cases of Neonatal Hemochromatosis	
3 cases of Methylmalonic Aciduria	

Table 3. Respiratory chain enzyme analysis

i)	<i>In vitro</i> respiratory chain enzyme assay
ii)	BN-PAGE in gel enzyme staining
iii)	BN-PAGE western blotting
iv)	Quantitative PCR

Fig. 1. The results of enzyme assay of respiratory chain in eight cases. Complex I and III were extremely low activities.

ratio mtDNA/nDNA using these samples (Fig. 3). They all exhibited extremely low ratio (2-7%; normal is 60-150%). Based on these data, they were diagnosed as mtDNA depletion syndrome. Thus, the present study showed that primary mitochondrial hepatopathies were mainly caused by mtDNA depletion.

In the metabolic liver diseases, all cases except MMA cases exhibited mildly or moderately suppressed activities of complex I, II and IV (also complex II in some cases). However, the respective protein levels remained almost normal in them (Figs. 4 and 5).

Unlike other metabolic diseases, MMA cases exhibited low activities and protein levels of complex I, III and IV as did patients with mtDNA depletion syndrome. In particular, their low activity and protein levels of complex

I was noticeable. Surprisingly, they all exhibited normal ratios of mtDNA to nDNA (Fig. 6 and Table 4). However, the mechanism underlying depleted RC proteins in an absence of depletion of mtDNA remains unclear.

The mitochondrial respiratory chain transfers electrons derived from oxidation of sugars, fats and proteins through a series of four enzyme complexes to generate an electrochemical proton gradient. ATP synthase, or complex V, then utilizes this gradient to derive ATP synthesis. This process of oxidative phosphorylation is responsible for bulk of ATP synthesis in virtually all tissue, so it is not surprising that RC dysfunction can affect any or all organ systems including liver (Thorburn, 2004; Munnich and Rustin, 2001).

Lactic acidemia is the classic biochemical finding of

Fig. 2. BN-PAGE and Western blotting in case1-4. The bands of complex I, III, and IV were significantly decreased.

Fig. 3. The ratio of mtDNA/nDNA by the real-time PCR. All cases are significantly low ratio of mtDNA (normal range; 40-150%).

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Fig. 4. The result of enzyme assay of respiratory chain in 12 cases with metabolic liver disease (OTC deficiency, Wilson disease, and Neonatal hemochromatosis).

Fig. 5. BN-PAGE and Western blotting in the cases with metabolic liver disease (OTC deficiency, Wilson disease, and Neonatal hemochromatosis).

Fig. 6. Metabolic pathway about methylmalonic aciduria.

Fig. 7. The result of enzyme activity and BN-PAGE analysis of respiratory chain in methylmalonic aciduria.

Table 4. The ratio of mtDNA/nDNA by the real-time PCR in methylmalonic aciduria

mtDNA/nDNA (% of control)		
Case 1 (F)	Case 2 (M)	Case 3 (F)
49	48	113

Normal range: 40-150%

mitochondrial disease, but Kirby *et al.* (1999) reported that blood lactic acid level was normal in about 20 percent of patients with complex I deficiency. About a half of our patient usually showed normal blood lactate level.

Recently, BN-PAGE immunoblotting and enzyme staining have been shown to be a powerful diagnostic tool for mitochondrial disorders. We confirmed their utility in studying these patients. More investigation is required to acquire a better understanding of liver disease mitochondrial respiratory chain disorders.

The present study strongly suggested that considerable disturbance of mitochondrial respiratory chain occurs in children with metabolic diseases and possibly modifies the pathophysiology of the liver disease.

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