Mutation of the Fumarase Gene in Two Siblings with Progressive Encephalopathy and Fumarase Deficiency

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Abstract

We report an inborn error of the tricarboxylic acid cycle, fumarase deficiency, in two siblings born to first cousin parents. They presented with progressive encephalopathy, dystonia, leucopenia, and neutropenia. Elevation of lactate in the cerebrospinal fluid and high fumarate excretion in the urine led us to investigate the activities of the respiratory chain and of the Krebs cycle, and to finally identify fumarase deficiency in these two children. The deficiency was profound and present in all tissues investigated, affecting the cytosolic and the mitochondrial fumarase isoenzymes to the same degree. Analysis of fumarase cDNA demonstrated that both patients were homozygous for a missense mutation, a G-955 → C transversion, predicting a Glu-319 → Gln substitution. This substitution occurred in a highly conserved region of the fumarase cDNA. Both parents exhibited half the expected fumarase activity in their lymphocytes and were found to be heterozygous for this substitution. The present study is to our knowledge the first molecular characterization of tricarboxylic acid deficiency, a rare inherited inborn error of metabolism in childhood. (*J. Clin. Invest.* 1994. 2514–2518.) Key words: encephalopathy · fumarase deficiency · fumarase cDNA · Krebs cycle · mitochondria.

Introduction

Among the causes of congenital lacticacidosis, inborn errors of oxidative phosphorylation and pyruvate metabolism are widely recognized and are well documented (1–3). However, little is known regarding the genetic defects of the tricarboxylic acid cycle, and only a few cases have been reported to date of which ~10 had fumarase deficiency (4,5).

Human tissues contain two fumarase isoenzymes, cytosolic and mitochondrial fumarases (6). A noticeable exception is brain, which in rat has been shown to contain only the mitochondrial enzyme (7). Human tissue fumarase is almost equally distributed between the mitochondria, where the enzyme catalyzes the reversible hydration of fumarate to malate as a part of the tricarboxylic acid cycle, and the cytosol, where it is involved in the metabolism of the fumarate released by the urea cycle. The two isoenzymes have quite homologous structures. In rat liver, they differ only by the acetylation of the NH_2-terminal amino acid of the cytosolic form (8). In all species investigated so far, the two isoenzymes have been found to be encoded by a single gene (9,10). This gene has been localized on chromosome 1 in humans (11,12). In rat, the two isoenzymes might be synthesised from a single mRNA by alternative translational initiation (13), whereas, in humans, they might originate from two distinct mRNA species by alternative splicing (14). None of the complete sequences of the putative cDNAs have been successfully obtained in humans. Nevertheless, the complete sequence of mature fumarase, identical for the mitochondrial and the cytosolic enzymes, has been reported (14), enabling mutation screening to be performed in cases of fumarase deficiency affecting both the cytosolic and mitochondrial isoenzymes.

Hitherto, clinical features reported in early stages of fumarase deficiency mainly involved neurological impairment and encephalopathy (15). Facial dysmorphism and an unusual neonatal liver disorder have also been noted in one patient and were tentatively attributed to the deficiency (16). Fumaric aciduria is consistently observed, associated with increased excretion of one or more of the Krebs cycle intermediates, succinic acid, α-ketoglutaric acid, citric acid, or malic acid (5,15). Metabolic acidosis was generally absent (or mild) in the blood before the terminal stages, when blood lactate was markedly raised (4). The clinical course can be static or fatal (< 2 yr) and this difference in outcome has been tentatively correlated with the level of residual enzyme activity or with differential tissue involvement (5).

This study reports on the first molecular characterization of fumarase deficiency affecting both isoenzymes in two sisters presenting with encephalopathy.

Case reports

Case 1. A girl was born to first cousin Moroccan parents after a term pregnancy and dystocic delivery due to excessive fetal weight. Neonatal parameters were unknown. Two siblings had died in the neonatal period under unexplained circumstances. One brother and two sisters are healthy. Limb movements were judged hypertonic in the first months of life. At two and one half years of age, she was severely retarded and abnormal movements persisted. She failed to thrive thereafter (weight - 2 SD, height - 2 SD) and gradually developed severe microcephaly (head circumference - 4 SD). At seven years, she is bedridden with major kyphosis, hyperextension of lower limbs and equin-

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ovarus deformation of feet. Motor activity in upper limbs was disturbed by bizarre postures and bursts of movements predominating on extensors. The Denver Developmental Test averaged 8 mo. Despite a severe motor disability, she can smile and follow with the eyes. MR imaging of the brain was normal. Blood count consistently showed neutropenia (810 polymorphonuclear cells/mm^3) with no anemia or thrombopenia. Histopathological examination of the liver showed mild vacuolization of hepatocytes and inflammatory infiltrates in interlobular areas. In skeletal muscle, abnormal variation in fiber diameter with predominance and hypertrophy of type II fibers was noted. The Gomori trichrome stain was normal.

Case 2. Her sister was born after a term pregnancy and normal delivery (birth weight, 3,700 g; head circumference, 36.5 cm; APGAR 10). Her mother was given insulin for late onset diabetes mellitus at 5 mo of pregnancy. Poor sucking, vomiting, and failure to thrive were noted in the first 2 wk of life and hypertonic episodes of the upper limbs were noted from the age of 6 mo. At 14 mo, she had moderate microcephaly (head circumference - 2.5 SD), trunk hypotonia, hypertonic and dystonic posture of the limbs, and developmental delay (Denver Developmental Test = 6 mo). Yet, her behavior was lively and cooperative and no dysmorphic features were noted. CT scan of the brain showed dilation of pericerebral spaces and ventricles and MR imaging (T2) revealed a slight increased signal intensity. Electromyographic, electroencephalographic, and electroretinographic studies were negative. The blood count showed severe neutropenia (300–600/mm^3). Serum ammonia and urinary orotic acid were within normal ranges, including after a 1 g/kg oral protein load. Liver function tests were normal (except for a fourfold increase in gamma glutamyl transferase). Histopathological examination of the liver and the jejunum were also normal, while changes in the skeletal muscle were similar to those found in case 1.

Methods

Lactate and pyruvate levels and their molar ratios were determined in plasma and cerebrospinal fluid by enzymatic methods as indexes of the oxidation reduction status (17). Amino acid levels were determined by using an automatic amino acid analyzer, and urinary organic acids were studied by gas chromatography–mass spectrometry (18).

Skeletal muscle homogenates and mitochondria-enriched fractions were prepared from open biopsies of the deltoid (120 mg) (19,20). Liver homogenates were prepared from needle biopsies (5 mg; reference 20). Circulating lymphocytes were isolated on a Ficoll cushion and B lymphoblastoid cell lines (BLCL)[1] were established from Epstein-Barr Virus–transformed lymphocytes. Cultured skin fibroblasts and BLCL were grown in RPMI 1640 (GIBCO BRL, Gaithersburg, MD) supplemented with 10% undialyzed fetal calf serum, 2 mM glutamine, 2.5 mM sodium pyruvate, 100 μg/ml streptomycin, 100 U/ml penicilin, and 200 μM uridine at 37°C under standard conditions (21).

Fumarase (EC 4.2.1.2), citrate synthase (EC 4.1.3.7), lactate dehydrogenase (EC 1.1.1.27), cytochrome *c* oxidase (EC 1.9.3.1), and succinate cytochrome *c* reductase activities were spectrophotometrically measured according to standard procedures (20, 22). Polarographic studies were performed in a 250 μl cell using 30 μg protein as described (20).

Total RNAs were extracted from either cultured skin fibroblasts or BLCL (23) and reverse transcribed using the GeneAmp® RNA PCR kit (Perkin-Elmer Corp., Norwalk, CT). The fumarase cDNA was amplified in overlapping fragments using four pairs of primers (fragment 1: primer 5′ nt 1-nt 21, primer 3′ nt 399-nt 379; fragment 2: primer 5′ nt 344-nt 364, primer 3′ nt 761-nt 741; fragment 3: primer 5′ nt 683-nt 703, primer 3′ nt 1148-nt 1128; fragment 4: primer 5′ nt 1079-nt 1099, primer 3′ nt 1445-nt 1425) (14). After 30 cycles of PCR (95°C, 30 s; 47°C, 30 s; 72°C, 1 min, 30s), amplification products were purified on a 2% low-melting-point agarose gel and recovered by heating for 5 min at 65°C. Direct sequencing was performed on an automatic DNA sequencer (Applied BioSystems 373A sequencer) using 3.2 pmol of the amplification primer and 100 ng DNA and 9.5 ml sequencing reaction mixture (Applied BioSystems, Inc., Foster City, CA).

Results

For both patients, blood lactate, pyruvate, and lactate/pyruvate molar ratios (L/P) were normal according to several determinations (Table I). However, cerebrospinal fluid lactate and pyruvate were consistently elevated (Table I). While several aminoacids (phosphoethanolamine, threonine, alanine, valine, serine, glycine) were occasionally slightly elevated in blood and urine of patients, aspartate + asparagine were consistently low in patients' blood, being normal in urine (Table I). Urinary organic acid analyses were performed at 6 yr and 12 mo of age for patients 1 and 2, respectively. They both revealed major excretion of fumaric acid and, to a lesser extent, of α-ketoglu-

Table I. Metabolic Investigations in Plasma, Cerebrospinal Fluid, and Urine of the Two Patients (P1, P2) and Controls (C)

	P1	P2	C
Plasma			
Organic acids (mmol/l)			
Lactate	1.2	1.6	0.6–2.4
Pyruvate	0.15	0.12	0.04–0.19
L/P ratio	7.5	8.0	<20
β-hydroxy butyrate	0.05	0.03	0.02–0.09
Acetoacetate	0.07	0.08	0.016–0.04
Ketone body ratio	0.71	0.37	<2
Amino acids (μmol/l)			
Aspartate	3	9	4–20
Asparagine	24	13	36–191
Glutamate	43	73	20–107
Glutamine	492	453	243–822
Cerebrospinal fluid			
Organic acids (mmol/l)			
Lactate	3.2	4.7	0.6–2.4
Pyruvate	—	0.3	0.04–0.19
L/P ratio	—	15.6	<20
Urine			
Organic acids (μmol/mmol creatinine)			
Fumarate	781	727	<16
α-ketoglutarate	325	99	<13
Amino acids (μmol/l)			
Aspartate	6	16	2–10
Asparagine	24	52	0–32
Glutamate	3	194	0–11
Glutamine	72		52–165

Experimental procedures as described in Methods.

1. *Abbreviations used in this paper:* BLCL, B lymphoblastoid cell lines; L/P, lactate/pyruvate ratio.

taric acid (Table I). Other tricarboxylic acid cycle intermediates, including succinic acid, were present in normal amounts (not shown).

Biochemical investigations were initially performed on isolated lymphocytes from the two patients and controls (Table II). Respiratory rates measured on patient's intact lymphocytes were similar to control rates (Table II). Likewise, succinate oxidation by detergent-permeabilized lymphocytes was linear and proceeded at a normal rate in both cases (not shown). Activities of respiratory chain enzymes (cytochrome *c* oxidase, succinate cytochrome *c* reductase) were also normal. However, under conditions where cytosolic lactate dehydrogenase and mitochondrial citrate synthase were freely measurable (cells and mitochondria lysed by 0.02 % Triton X-100), the rate of fumarase activity in lymphocytes was < 0.5% of that of controls in both patients. Measurement of fumarase activity using mixed extracts from patient and control cells ruled out the presence of any inhibitory compound in the patients' extracts (not shown). These results suggest that both cytosolic and mitochondrial fumarase were markedly deficient in these two patients.

Table II. Enzyme Activities in Cells and Tissues of Patients (P1, P2) and Controls (C)

	P1	P2	C
Circulating lymphocytes			(n = 10)
Oxygen uptake (nmol O_2/min/mg protein)	4.8	5.6	2.5–6.4
Enzyme activities (nmol/min/mg protein)			
Cytochrome c oxidase	97	116	58–191
Succinate cytochrome c reductase	33	29	20–64
Fumarase	<0.1	<0.1	27–39
Citrate synthase	102	134	86–145
Lactate dehydrogenase	1340	1200	906–1550
Citrate synthase/fumarase ratio	>1000	>1000	2.8±0.3*
Skeletal muscle			(n = 6)
Enzyme activities (nmol/min/mg protein)			
Fumarase	0.12	0.15	77–90
Citrate synthase	196	104	155–194
Citrate synthase/fumarase ratio	1633	693	2.0±0.2
Cultured skin fibroblasts			(n = 5)
Enzyme activities (nmol/min/mg protein)			
Fumarase	<0.1	<0.1	103–125
Citrate synthase	75	100	140–173
Citrate synthase/fumarase ratio	>1000	>1000	1.4±0.1
Liver			(n = 5)
Enzyme activities (nmol/min/mg protein)			
Fumarase	0.15	—	71–92
Citrate synthase	69	—	126–161
Citrate synthase/fumarase ratio	460		1.7±0.2

Values are the mean of three determinations. Experimental procedures as described in Methods. * Mean value±SD.

Further investigations, carried out on skeletal muscle homogenates from both patients, indicated a profound reduction in the fumarase activity (< 0.5% of controls; Table II). Polarographic studies on isolated intact mitochondria from the skeletal muscle of patient 2 revealed a rapid inhibition of succinate oxidation (not shown). This could be ascribed to the inhibition of the succinate dehydrogenase by the fumarate accumulated in the mitochondrial matrix space, along with succinate oxidation. In agreement with this, progressive inhibition of the enzyme was not observed when the activity of the succinate dehydrogenase was measured in disrupted mitochondria in which fumarate accumulation did not occur (not shown).

Finally, fumarase was also found to be markedly deficient (< 0.5% of controls) in liver homogenate from patient 1, and in cultured skin fibroblasts from both patients.

Similar biochemical studies carried out on lymphocytes from both parents showed a 50% decrease in fumarase activity compared to control values. To assess the true heterozygosity of the parents for both isoenzymes, measurements were performed in the cytosolic and mitochondrial compartments of parents' BLCL (Table III). While citrate synthase activity levels were similar in the parents' and controls' sub-cellular compartments, fumarase represented ~ 50% of mean control values in both the mitochondrial and the cytosolic compartments in the parents, indicating that cytosolic and mitochondrial isoenzymes were similarly affected.

Sequence analysis of the common domain of cytosolic and mitochondrial fumarase cDNA from both patients' cultured fibroblasts and BLCL revealed a base substitution on comparison with our control sequence and with the available published

Table III. Enzyme Activities in Cytosolic and Mitochondrial Fractions of B-Lymphoblastoid Cell Lines From Patient 1 (P1), Parents, and Controls (C)

	P1	Mother	Father	Controls
Circulating lymphocytes				(n = 10)
Enzyme activities (nmol/min/mg protein)				
Fumarase	<0.1	14 (42%)*	19 (57%)	27–39
Citrate synthase	134	66	92	86–145
Citrate synthase/fumarase ratio	>1000	4.7	4.8	2.8±0.3‡
B lymphoblastoid cell lines				
Enzyme activities (nmol/min/mg protein)				(n = 6)
Cytosolic compartment				
Fumarase	<0.1	8 (47%)	7.5 (44%)	17–23
Citrate synthase	0	0	0	0
Lactate dehydrogenase	1102	1050	984	900–1250
Mitochondrial compartment				
Fumarase	<0.1	8 (44%)	9 (50%)	16–20
Citrate synthase	30	40	30	31–40
Citrate synthase/fumarase ratio	>300	5	3.3	2.1±0.3
Whole cell				
Fumarase	<0.1	16 (46%)	16.5 (47%)	33–40
Citrate synthase	30	40	30	31–40
Lactate dehydrogenase	1102	1050	984	900–1250
Citrate synthase/fumarase ratio	>300	2.5	1.8	1.0±0.2

Values are the means of three determinations. Experimental procedures are described in Methods. * Percent of control mean; ‡ mean value±1 SD.

sequence (14). This consisted of a G → C transversion at position 955, which changed the GAA codon for Glu-319 to a CAA codon for Gln (E319Q) (Fig. 1 A). This change from an acidic to a neutral residue occurred in a highly conserved domain of the fumarase protein (Fig. 1 B). Fumarase cDNAs from both parents were sequenced directly and were found to be heterozygous for the G955C transversion (Fig. 1 A).

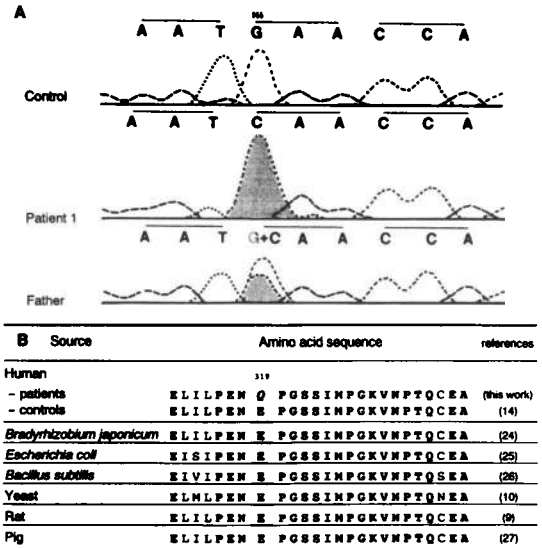
Moreover, several discrepancies with the previously published human fumarase gene sequence were observed. They were all in the homozyggous state and present in one Italian and one French control, as well as in patients. Several of these resulted in amino acid changes: a G → C transversion at position 692 (R231A) and a C → G transversion at position 748 (L250V). Moreover, successive base substitutions were noted at position 863–865, modifying successive amino acids: a GC transversion at nt 863, a C → T transition at nt 864 and a T → G transition at nt 865 (S288T, S289A). Finally, 3 nt (TGC) from nt 1,008 to 1,010 were absent from our sequences, removing an alanine residue from the protein sequence at codon 337. All these amino acid substitutions and the loss of the alanine residue provided further evidence of homology between the protein sequence of human fumarase and that of other species (9,10,24–27). Three base substitutions, also present in controls' and patients' cDNA, did not affect the nature of the amino acids: a G → C transversion at nt 132, a C → G transversion at nt 691 and a A → C transversion at nt 858.

Discussion

This study reports on fumarase deficiency in two sisters with a similar picture of psychomotor retardation, failure to thrive, microcephaly, and abnormal posture with axial hypotonia contrasting with hypertonia of limbs, all suggestive of a progressive metabolic encephalopathy. The observation of permanent hyperlactatorachia in the two siblings born to first cousin parents was highly suggestive of a recessively inherited congenital lactic acidosis. This condition can be explained by distinct mechanisms: inborn errors of oxidative phosphorylation or genetic defects of the tricarboxylic acid cycle. Investigation of the oxidoreduction status in plasma did not enable us to discriminate between these two conditions in our patients. Little is known regarding the oxidoreduction profiles in tricarboxylic acid cycle disorders as few patients with this condition have been reported. However, it is noteworthy that hyperlactatemia has been consistently reported in patients presenting with other Krebs cycle defects, such as succinate dehydrogenase (28,29) or α-ketoglutarate dehydrogenase deficiencies (30). In most cases of fumarase deficiency, no hyperlactatemia (4,16) could be observed before the terminal stages (4). One can speculate that blockade of the Krebs cycle resulting from the virtual absence of both mitochondrial and cytosolic fumarase activities can be bypassed in most human tissues, except brain, by a metabolic pathway that remains to be elucidated. In our patients, the low aspartate plus asparagine concentration consistently measured in both patients' blood might reflect an active transamination of α-ketoglutarate which can create a shunt to produce oxaloacetic acid, schematized in Fig. 2. It is also noteworthy that no impairment of the urea cycle due to the cytosolic fumarase deficiency was noted in our patients, as indicated by the normal serum ammonia and urinary orotic acid levels.

The severe fumarase deficiency (< 0.5% of control activity), present in all tissues investigated, affected the mitochondrial and the cytosolic isoenzymes to a similar degree. In keeping with this, a direct relationship between the severity of the defect, its widespread distribution among tissues and the fatal course of the disease, was not observed in our patients (one is still alive at 7 yr of age).

In all cases reported so far, including ours, fumarase deficiency predominantly affected brain function (4,5,14,15,31,32). Indeed, despite the widespread expression of the cytosolic and mitochondrial fumarase deficiency in our patients, other organs known to require a high energy level to function and gener-



B Source	Amino acid sequence	references
Human	319	
- patients	ELILPEN Q PGSSINPGKVNPTQCEA	(this work)
- controls	ELILPEN E PGSSINPGKVNPTQCEA	(14)
Bradyrhizobium japonicum	ELILPEN E PGSSINPGKVNPTQCEA	(24)
Escherichia coli	EISIPEN E PGSSINPGKVNPTQCEA	(25)
Bacillus subtilis	EIVIPEN E PGSSINPGKVNPTQSEA	(26)
Yeast	ELMLPEN E PGSSINPGKVNPTQNEA	(10)
Rat	ELILPEN E PGSSINPGKVNPTQCEA	(9)
Pig	ELILPEN E PGSSINPGKVNPTQCEA	(27)

Figure 1. Sequence analysis of fumarase cDNA from a control, patient 1 and the father of patient 1 (*A*). Multiple sequence alignment of the highly conserved domain of the fumarase protein from patients, controls and from different species (*B*).

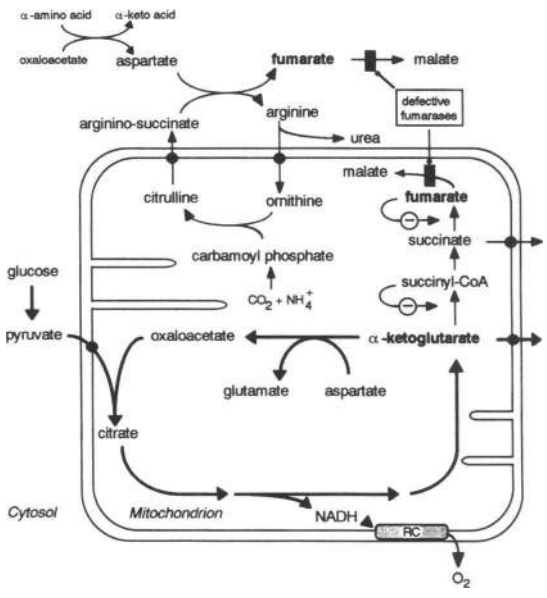


Figure 2. Hypothetical scheme of mitochondrial tricarboxylic acid metabolism in the patients' cells. The blockade of the tricarboxylic acid cycle due to fumarase deficiency may be partially released by the transamination of the α-ketoglutarate producing oxaloacetate, thus allowing pyruvate oxidation in the mitochondrial matrix. *RC*, respiratory chain.

ally involved in mitochondrial disorders (1), *i.e.*, optic nerve and skeletal muscle, were not clinically affected.

Why brain is specifically involved in patients with fumarase deficiency remains unclear. Besides a high energy requirement for normal functioning, the brain might have a particular metabolic organization accounting for such involvement. During this study, we had the opportunity to investigate frozen *post-mortem* brain tissue from two controls and found that citrate synthase, a mitochondrial matrix enzyme, and fumarase were similarly distributed in subcellular compartments, suggesting the absence of a cytosolic form of fumarase in human brain, as previously reported in rat brain (7).

In our patients, molecular investigation revealed a single base substitution at nucleotide 955, as shown by direct sequencing of the fumarase cDNA from the patients' fibroblasts and BLCL. The two patients were found to be homozygous for this substitution. No other substitutions were identified on comparison with controls. The two consanguineous parents, whose fumarase activity was about half of control levels, were found to be heterozygous for this base substitution. The GC transversion at nucleotide 955 replaced an acidic residue, Glu-319 (GAA), by a neutral residue, Gln (CAA). This change occurred in a highly conserved domain of fumarase which has been proposed as a candidate for the active site of the enzyme (27). Moreover, it is noteworthy that acidic residues, and in particular a glutamyl residue, have been shown to be involved in the active site of the enzyme (33). These results support the hypothesis that this mutation is responsible for the fumarase deficiency. To our knowledge, this is the first mutation described in fumarase cDNA and more generally in a Krebs cycle enzyme gene. Expression studies using mutant and revertant fumarase cDNA should allow better characterization of the role of the Gln for Glu-319 substitution in enzyme catalysis.

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