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RESEARCH ARTICLE

Molecular and Phenotypic Heterogeneity in Mitochondrial Trifunctional Protein Deficiency Due To β-Subunit Mutations

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The mitochondrial trifunctional protein (TFP) is a multienzyme complex of the fatty acid β-oxidation cycle. It is composed of four α-subunits (HADHA) harboring long-chain enoyl-CoA hydratase and long-chain L-3-hydroxyacyl-CoA dehydrogenase (LCHAD) and four β-subunits (HADHB) harboring long-chain 3-ketoacyl-CoA thiolase (LKAT). Mutations in either subunit can result in TFP deficiency with reduced activity of all three TFP enzymes. We characterize 15 patients from 13 families with β-subunit mutations by clinical, biochemical, and molecular features. Three clinical phenotypes are apparent: a severe neonatal presentation with cardiomyopathy, Reye-like symptoms, and early death (n=4); a hepatic form with recurrent hypoketotic hypoglycemia (n=2); and a milder later-onset neuromyopathic phenotype with episodic myoglobinuria (n=9). Maternal HELLP syndrome occurred in two mothers independently of the fetal phenotype. Mutational analysis revealed 16 different mutations, the majority being missense mutations (n=12). The predominance of missense mutations and the milder myopathic phenotype are consistent. Based upon homology to yeast thiolase that has been characterized structurally, the mutation localization within the protein correlates with the clinical phenotype. Outer loop mutations that are expected to alter protein stability less were only present in milder forms. The degree of reduction in thiolase antigen also correlated with the severity of clinical presentation. Although TFP deficiency is highly heterogeneous, there is genotype–phenotype correlation. Hum Mutat 21:598–607, 2003.

KEY WORDS: fatty acid oxidation; TFP; MTP; cardiomyopathy; Reye syndrome; neuropathy; myoglobinuria; HADHA; HADHB; LCHAD

DATABASES:
HADHA – OMIM: 600890; GDB: 434026; GenBank: NM_000182 (mRNA)
HADHB – OMIM: 143450, GDB: 344953; GenBank: NM_000183 (mRNA)

INTRODUCTION

Mitochondrial β-oxidation plays a major role in energy production, especially during periods of fasting. More than 20 enzyme and transport defects of the mitochondrial oxidation of saturated fatty acids are known [Gregersen et al., 2001; Rinaldo et al., 2002]. Clinically, fatty acid oxidation (FAO) disorders are heterogeneous with cardiomyopathy, hepatopathy, skeletal myopathy, and hypoketotic hypoglycemia as recognized features. Defects affecting the mitochondrial trifunctional protein (TFP) are not well characterized yet.

TFP is a multienzyme complex composed of four α-subunits (HADHA; MIM# 600890) containing the long-chain 2-enoyl-CoA hydratase and the long-chain L-3-hydroxyacyl-CoA dehydrogenase (LCHAD) domains and four β-subunits (HADHB; MIM# 143450) containing the long-chain 3-ketoacyl-CoA thiolase (LKAT) domain. For long-chain fatty acid substrates,

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these enzymes catalyze the last three steps of the mitochondrial β-oxidation.

TFP α- and β-subunits are encoded by separate nuclear genes located in the same region of chromosome 2p23 [Yang et al., 1996]. Since 1989 when Wanders et al. [1989] described a child with sudden infant death syndrome (SIDS) and accumulation of long-chain 3-hydroxy-fatty acids, TFP defects have been molecularly characterized [Wanders et al., 1992; Brackett et al., 1995; Ushikubo et al., 1996; Wanders et al., 1999; den Boer et al., 2002]. Two categories of diseases of the TFP complex are distinguished: isolated LCHAD deficiency (LCHADD) and general TFP deficiency. TFP deficiency is defined by reduced activity of all three TFP enzymes. Isolated LCHAD deficiency is defined by reduced LCHAD activity. The other two TFP enzymes might also be affected in LCHADD, but their activities are usually greater than 60% of normal. More than 60 patients with isolated LCHADD have been reported [Wanders et al., 1999; den Boer et al., 2002]. Disease is associated with the common α-subunit mutation 1528G>C, E474Q that is located directly within the catalytic region of the LCHAD domain. In 1992, the first patient with biochemical evidence of deficiency of all three TFP enzymes was described [Wanders et al., 1992], and in 1995, the first α-subunit mutations causing this disorder were defined [Brackett et al., 1995]. These were mostly frameshift, nonsense, or splice-site mutations resulting in exon skipping [Ushikubo et al., 1996; Ibdah et al., 1999].

To date, 15 patients from 12 families with TFP deficiency due to α-subunit mutations are known [Ushikubo et al., 1996; Schaefer et al., 1996; Isaacs et al., 1996; Ibdah et al., 1998; Ibdah et al., 1999; Matern et al., 1999; Hintz et al., 2002; three patients unpublished]. In contrast, only four patients with TFP deficiency due to β-subunit mutations have been reported [Ushikubo et al., 1996; Orii et al., 1997]. Clinical information on patients with β-subunit mutations is minimal and the molecular basis of this disorder is unknown.

We characterize here 15 patients from 13 different families with TFP deficiency and β-subunit mutations with regard to clinical features, type of mutation, mutation localization within the thiolase protein and thiolase protein expression, and focus on genotype–phenotype correlation in this TFP deficiency subgroup.

MATERIALS AND METHODS

Patients

Fourteen patients included in this study presented with clinical symptoms suggestive of a fatty acid oxidation disorder. One individual was identified by prenatal diagnosis. Patients' clinical and family histories were obtained from the referring physicians. One patient [Ushikubo et al., 1996], whose clinical features were incorrectly described previously, is also included in this report to provide longer-term follow-up.

Because all patients were diagnosed and evaluated in different metabolic centers, there were no common diagnostic and treatment protocols.

Eleven patients were biochemically characterized by LCHAD and LKAT enzyme assays in cultured fibroblasts derived from skin biopsies. Reduced LCHAD and LKAT activities were detected in all. Prenatal enzyme analysis in placenta confirmed the diagnosis in Patient 9; his brother (Patient 8) was previously identified as having TFP deficiency. We performed molecular genetic analysis of the TFP α- and β-subunit genes in all 15 patients. All studies were performed with the approval of the Vanderbilt University Medical Center or Washington University Institutional Review Boards.

Enzyme Analysis

We measured LKAT and LCHAD activities in fibroblasts according to previously reported techniques [Wanders et al., 1999]. Healthy individuals served as controls.

Western Blot Analysis

Western blot analysis was performed following 4 to 20% SDS-PAGE [Laemmli, 1970] using rabbit polyclonal antibodies raised against the β-subunit thiolase domain. A healthy individual served as control.

Cell Lines and DNA Isolation

Fibroblast cell lines were maintained in Dulbecco's modified Eagle's medium (GIBCO, www.invitrogen.com) with 10% fetal bovine serum, 20 mmol/L glutamine, antibiotics, and non-essential amino acids. DNA was isolated from whole blood or fibroblasts by standard techniques [Ausubel et al., 1994].

Polymerase Chain Reaction Amplification and Single-Stranded Conformational Variance Analysis

All 20 α-subunit and all 16 β-subunit exons of patients' genomic DNA were amplified in the presence of (^{32}P)-dCTP by the polymerase chain reaction (PCR) under standard conditions [Ausubel et al., 1994]. Details about the amplification of α-subunit exons, including oligonucleotide sequences of the primer pairs, have previously been reported [Brackett et al., 1995]. The α-subunit PCR products were analyzed by single-stranded conformational variance (SSCV) [Orita et al., 1989]. For the amplification of β-subunit exons, we designed 23- to 27-bp-long intronic primer pairs based upon our own and previously reported genomic thiolase DNA sequences [Kamijo et al., 1994]. These primer sequences are available on request. β-subunit exon pairs (2 and 3, 8 and 9, 11 and 12, and 14 and 15) were amplified together.

Sequencing Analysis

We sequenced exonic DNA of the α-subunit exhibiting altered band mobilities on SSCV analysis compared with amplified control DNA. All 16 β-subunit exons were directly sequenced using the reported intronic primer pairs. For sequencing analysis of exon 4, a nested oligonucleotide primer was used.

RESULTS

Clinical Features of TFP-Deficient Patients

Fifteen patients from 13 different families with documented TFP β-subunit mutations were included

TABLE 1. Clinical Features of TFP-Deficient Patients With β-Subunit Mutations*

Patient	Sex	Origin	Age at onset	Hypo-glycemia	Cardio-myopathy	Skeletal myopathy	Neuropathy	HELLP syndrome	Current age	Current state	Phenotype
1	♀	T	5d	+	+	–	–	+	Died 8d		Lethal
2	♂	C	1d	+	+	–	–	–	Died 12d		Lethal
3	♀	C	2d	+	+	–	–	–	Died 9d		Lethal
4	♂	C	4d	+	+	–	–	–[b]	Died 14d		Lethal
5	♂	C	5m	+	–	–	–	–	1y 5m	Asympt.	Hepatic
6	♂	C	2d	+	–	–	–	–	2y 2m	Delayed	Hepatic
7	♀	C	3y	+	–	+	+	–	23y	N	Myopathic
8[d]	♂	H	1y	–	–	+	+	–	4y	N, M	Myopathic
9[d]	♂	H	1y	–	–	+	+	–	1y 7m	N, M	Myopathic
10[e]	♂	I	1-2y	–	–	+	+	–	15y	N, M	Myopathic
11[e]	♂	I	1-2y	–	–	+	+	–	12y	N, M	Myopathic
12	♂	C	6y	–	–	+	+[a]	–	10y	N, M	Myopathic
13	♂	C	3y	–	+[c]	+	+[a]	–	25y	N, M	Myopathic
14	♀	C	3y	–	–	+	n.a.	–	11y	M	Myopathic
15	♂	C	1-2y	–	–	+	n.a.	+	3y 4m	M	Myopathic

*Patients 1 through 15 identify the same individuals in all figures and tables; Age: d, day; m, month; y, year; T, Turkish; H, Hispanic; I, Israeli; C, Caucasian; N, peripheral neuropathy; M, skeletal myopathy; n.a., not available.
[a]Axonal and myelin degeneration documented by sural nerve biopsy.
[b]Preeclampsia of mother terminated pregnancy early.
[c]During metabolic decompensation.
[d]Brothers.
[e]Brothers.

in this study (Table 1). There were four girls and eleven boys. The age at disease-onset ranged from the first day to six years of life. The age at diagnosis was between the neonatal period and 18 years of life. In patients with the mildest phenotype, TFP deficiency was often documented years after the first clinical symptoms occurred.

Three different clinical phenotypes are apparent. Within days after birth, four newborns (Patients 1–4) presented with severe dilated cardiomyopathy that was diagnosed with standard echocardiographic criteria for abnormal left ventricular systolic performance and structure, lactic acidosis, and a Reye syndrome-like picture of hypoketotic hypoglycemia. All of these patients died in the neonatal period. Cardiac pathology in one patient (Patient 1) revealed severe fibrosis without signs of inflammation and only moderate fatty infiltration on electron microscopy. We defined this presentation as the lethal phenotype.

Two patients (Patients 5 and 6) were diagnosed with a less severe manifestation characterized by recurrent hypoketotic hypoglycemia and lethargy during illness or periods of fasting. Onset of symptoms in both patients was in the first months of life. We defined this presentation as the hepatic phenotype. Both patients did not develop signs of cardiomyopathy, myopathy, or neuropathy during 18 months of follow-up. At last report, one patient was healthy and normally developed. The other suffered from a cardiorespiratory arrest during an infection and had to be resuscitated, an event resulting in severe motor and cognitive delay.

The third and largest group of patients (n=9, Patients 7–15) is characterized by a milder phenotype, with onset at age 1 to 6 years. We defined this as the neuromyopathic phenotype. The main features of this presentation were progressive peripheral neuropathy and episodic myoglobinuria. Peripheral neuropathy with progression of muscle weakness, was diagnosed clinically in seven patients, in two muscles, weakness alone was documented. Muscle biopsies in three patients (Patients 12–14) confirmed muscle fiber atrophy consistent with a denervating process. Sural nerve biopsies demonstrated axonal and myelin degeneration in three patients (Patients 10, 12, and 13). Shortness of breath, intermittent oxygen dependence, and hypoventilation during metabolic decompensation were observed in three patients (Patients 7, 12, and 13), suggesting weakness of the diaphragm. Exercise or illness-induced episodes of rhabdomyolysis were characterized by greatly elevated serum creatine kinase (CK) concentrations of 16,000 to 60,000 IU/L (Patients 10 and 12–15). CK normalized between episodes. Cardiomyopathy and episodes of hypoketotic hypoglycemia were generally not observed in this phenotype. However, Patient 13 developed recurrent moderate cardiomyopathy diagnosed by standard echocardiographic criteria during periods of metabolic decompensation that was completely reversible between episodes.

Follow-up through adolescence was possible in Patients 10, 11, 12, and 14 and through adulthood in Patients 7 and 13. All six individuals are in fairly stable clinical condition, but they suffer from episodic muscle pain, weakness, and exercise intolerance. During periods of illness, the myopathy worsens and myoglobinuria occurs. In Patient 7, progression of peripheral neuropathy has resulted in immobility at the age of 21 years.

Maternal liver disease during pregnancy is a complication associated with LCHAD deficiency in the fetus [Sims et al., 1993; Wilcken et al., 1993; Ibdah et al., 1999; den Boer et al., 2002]. It has occurred in two of twelve families with TFP deficiency due to α-subunit mutations [Isaacs et al., 1996; own data] and in another three families not characterized by mutational analysis [Chakrapani et al., 2000]. In our study, hemolysis, elevated liver enzymes, low platelets (HELLP) syndrome was observed in two of 13 mothers carrying a fetus with TFP deficiency and β-subunit mutations (Patients 1 and 15). The occurrence of HELLP syndrome was not dependent on the severity of the baby's phenotype. Patient 1 presented with the severe neonatal phenotype and died shortly after birth. Patient 15 had a milder neuromyopathic form of TFP deficiency with onset of first symptoms in infancy.

LCHAD and LKAT Activities in Fibroblasts

For biochemical characterization, enzyme activities of LCHAD (encoded on the α-subunit) and LKAT (encoded on the β-subunit) were measured in fibroblasts (Table 2). Normal data represent age-matched individuals without fatty acid oxidation disorders (n=5 in each case). In Patient 9, enzyme assays were performed in a placental extract. Fibroblasts from Patients 4 and 11 were not available for enzyme studies. The patients in this study were collected over a number of years and assays in patients and batch normals were performed at different times. LCHAD activities ranged from 10 to 41% of normal (mean 24%, SD±10%), and LKAT activities were 0 to 37% of normal (mean 10%, SD±9%). Patients with the severe phenotype resulting in neonatal death did not present with lower enzyme activities in fibroblasts than patients with the milder phenotypes. Enzyme assays in highly oxidative tissues, such as heart or skeletal muscle, were not performed.

TABLE 2. Biochemical Features in TFP-Deficient Patients With β-Subunit Mutations*

Patient	LCHAD sample	% of norm	LKAT sample	% of norm
1	9.1	15	4.7	16
2	17.6	29	0	0
3	20.1	34	1.0	3.4
4	n.a.		n.a.	
5	17.2	29	5.1	17
6	13.7	23	4.5	15
7	13.4	22	1.3	4.4
8	15.7	26	10.9	37
9	5.9[a]	18	0.9[a]	5
10	6.5	11	2.5	8.4
11	n.a.		n.a.	
12	6.2	10	0.01	0
13	8.6	14	3.3	11
14	13.5	23	0.8	2.7
15	24.4	41	4.9	17

*Patients 1 through 15 identify the same individuals in all figures and tables. LCHAD and LKAT activities were determined in fibroblast cells and measured in nmol/min/mg protein. Patients were collected over a number of years and assays in patients and batch normals were performed at different times. In batch normals, LCHAD activities ranged from 43.6 to 89.9 nmol/min/mg protein (mean: 60.2 ±13.2) and LKAT activities from 16.4 to 43.0 nmol/min/mg protein (mean: 29.6 ±7.4).
[a]In patient 9, enzyme activities were determined in placenta (LCHAD norm: 32.7 nmol/min/mg protein; LKAT norm: 18.1 nmol/min/mg protein).
n.a., not available.

The enzyme activities in fibroblasts clearly demonstrate TFP deficiency affecting both subunits. However, the residual activities are relatively high in many patients and might be due to other enzymes with overlapping substrate specificity in our in vitro assay system. Additionally, the enzyme assays are not mitochondria-specific, and enzymes from other organelles may contribute to the activities measured. The wide range of variability in the control assays might be explained by varying assay conditions, because studies were performed over a number of years. Although enzyme assays do not predict or correlate with the severity of the clinical phenotype, reduced activities adequately define the defect biochemically and correctly predict the appropriate genes for molecular analysis.

Western Blot Analysis

In addition to the β-subunit LKAT enzyme assay, we analyzed the β-subunit protein expression in 10 available fibroblast cell lines by Western blot (Fig. 1). Using the computer program Scion Image© (www.scioncorp.com), we compared thiolase protein antigen in each patient with thiolase expression of a healthy control individual. In three samples (Patients 2, 3, and 6), thiolase protein was barely detectable on immunoblot analysis (2–3% of control) and two of these patients presented with the severe phenotype (Patients 2 and 3). In four samples (Patients 5, 7, 8, and 12), reduction of protein levels was substantial, but antigen was readily detectable (7–8% of control). A moderate reduction in thiolase protein occurred in the remaining three patients (Patients 10, 13, and 14) (14–25% of control) who all had a mild phenotype.

Delineation of β-Subunit Mutations From Genomic PCR

Molecular diagnosis was done in all 15 patients with clinical and biochemical features consistent with TFP deficiency. SSCV analysis of amplified PCR products and DNA sequencing excluded α-subunit exonic mutations and mutations in the intronic splice sites in all patients. DNA sequencing of the β-subunit revealed 16 sequence differences in the thiolase exonic coding regions that alter amino acids or introduce a termination codon (Tables 3 and 4). These likely represent disease-causing mutations and are not present in normal individuals (n=100). β-subunit mutations in both alleles were defined in 10 patients including one homozygous individual

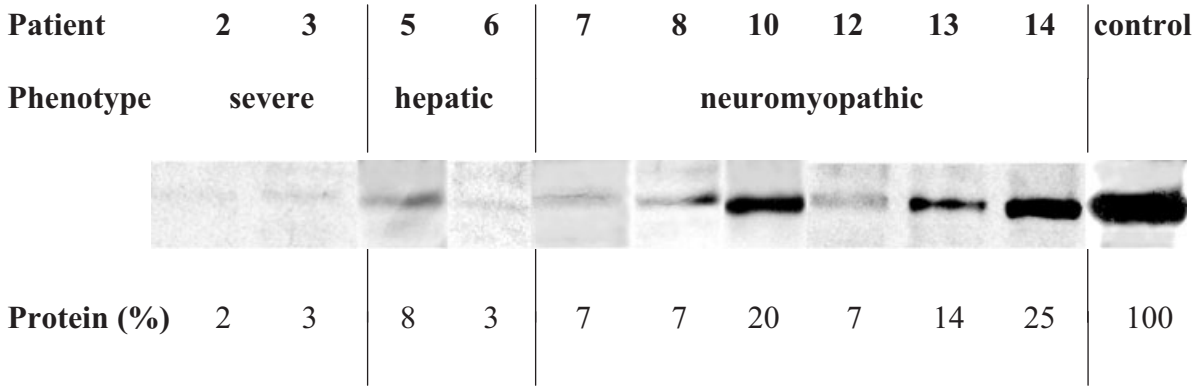

FIGURE 1. Western blot analysis of thiolase protein in fibroblasts. Patients 1 through 15 identify the same individuals in all figures and tables. In Patients 1, 4, 9, 11, and 15, fibroblast cell lines were not available. An individual with normal TFP activities served as the control. The amount of protein expressed was quantified with the computer program Scion Image © and is given in % of the control. The clinical phenotypes are labeled severe, hepatic, and neuromyopathic as described in the text and Table 1.

TABLE 3. β-Subunit Mutations in Patients With Trifunctional Protein Deficiency*

	Allele 1			Allele 2		
Patient	Exon	Mutation	Amino acid change	Exon	Mutation	Amino acid change
1	4	181C>T	R28C	4	181C>T	R28C
2	9	685C>T	R196X			
3	4	182G>A	R28H			
4	8	527C>G	S143X			
5	9	693delC	198 frameshift	10	881C>G	**P261R**
6	9	725A>G	**D209G**	10	839G>A	**G247D**
7	4	182G>A	R28H	9	740G>A	**R214H**
8	4	181C>T	R28C	6	349A>G	R84G
9	4	181C>T	R28C	6	349A>G	R84G
10	8	607C>T	R170X	10	881C>T	**P261L**
11	8	607C>T	R170X	10	881C>T	**P261L**
12	7	362T>C	L88P			
13	4	176G>A	G26D	9	740G>A	**R214H**
14	10	901G>A	**G268S**			
15	7	397A>C	T100P	10	881C>G	**P261R**
a Lit[a]	9	788A>G	**D230G**			
b Lit[a]	4	182G>A	R28H	9	740G>A	**R214H**
c Lit[b]	9	776-811del	226-237del	15	1331G>A	**R411K**
d Lit[b]	15	1331G>A	**R411K**	15	1331G>A	**R411K**

*Patients 1 through 15 identify the same individuals in all figures and tables. Patient 7 and patient b Lit [a] are compound heterozygous for exactly the same mutations and are the same individual. Mutations are designated by the nucleotide number from the start codon and the amino acid number from the mature N-terminus. Outer loop mutations, as further explained in the text, are written in bold letters.
[a]Ushikubo et al. [1996].
[b]Orii et al. [1997].

(Patient 1). In five patients, we found only a single mutation, even though direct sequence analysis of all 16 exons was done (Table 3). The predicted but undefined second mutations may occur in intronic regions crucial for thiolase gene expression that were not present in our amplified products. Additionally, we observed six β-subunit polymorphisms some of these were previously reported [Ushikubo et al., 1996]. The two in coding regions are in exon 2 (-32insT) that adds a threonine residue to the transit peptide and exon 10 (825T>C) that does not change the codon of V242. Four polymorphisms lie within intronic regions, especially between exons 11 and 12 (IVS11+66A>G, IVS11–12G>A, IVS11–14A>G, IVS14–87A>G).

Type of β-Subunit Mutation, Molecular Heterogeneity and Genotype–Phenotype Correlation

Mutational analysis revealed 12 different missense mutations at 10 different nucleotide positions, three premature termination mutations (R170X, S143X, R196X), and one frameshift mutation as the result of a one base pair deletion (693delC) (Table 4). Six missense mutations resulted in changes in amino acid

TABLE 4. β-Subunit Mutational Spectrum in Trifunctional Protein Deficiency

Mutation	Amino acid change	Exon	Mutant allele number
176G>A	G26D	4	1
181C>T	R28C	4	3 (4[a])
182G>A	R28H	4	2
349A>G	R84G	6	1 (2[a])
362T>C	L88P	7	1
397A>C	T100P	7	1
725A>G	D209G	9	1
740G>A	R214H	9	2
788A>G[b]	D230G	9	1
839G>A	G247D	10	1
881C>G	P261R	10	2
881C>T	P261L	10	1 (2[a])
901G>A	G268S	10	1
1331G>A[c]	R411K	15	3
527C>G	S143X	8	1
607C>T	R170X	8	1 (2[a])
685C>T	R196X	9	1
693delC	198 frameshift	9	1
776_811del[c]	226_237del	9	1

*Fourteen different missense mutations in the TFP β-subunit were delineated, including two reported in the literature. Three premature termination and two frameshift mutations, one of them previously reported, have been found.
[a]Including related patients.
[b]Reported by Ushikubo et al. [1996].
[c]Reported by Orii et al. [1997].

charge (G26D, R28C, R84G, D209G, G247D, P261R). In two patients (Patients 12 and 15), proline is substituted for leucine and threonine, respectively, an alteration that is predicted to interrupt helical structures. Four patients presented with a proline to arginine or leucine change (P261R, P261L). These alterations may affect secondary protein structure by extension of helices.

Nine patients were compound heterozygous for β-subunit mutations. A homozygous mutation (Patient 1) was detected in a Turkish patient of consanguineous parents. In five patients, only a single heterozygous mutation was delineated despite sequencing of all 16 β-subunit exons. Twelve mutations were found once (12 of 16). Five unrelated patients shared missense mutations at positions 181-182 in exon 4, making this site a mutational hot spot. A missense mutation in exon 9 (G740A) and one in exon 10 (C881G) occurred in two unrelated families. Two different missense mutations (C881G, C881T) appeared at the same position on exon 10 in three unrelated patients, again suggesting this as a mutational hot spot. Including the three other patients reported in the literature, exon 4 mutations were found on six alleles, exon 9 mutations on seven alleles, and exon 10 mutations on five alleles. Overall, these data demonstrate the genetic heterogeneity of TFP deficiency due to β-subunit mutations.

We detected the same mutations at positions 181–182 in exon 4 in patients with the severe lethal phenotype and in patients with predominantly milder skeletal myopathy. Two of the premature termination mutations were present in patients with the severe phenotype, the third in two brothers with neuromyopathy. The second allele in these two brothers carried a milder missense mutation consistent with the less severe phenotype. The frameshift mutation was also found in a patient with a milder phenotype, whose second allele carried a milder missense mutation consistent with the clinical phenotype. Thus, in compound heterozygote individuals with a missense mutation on one allele and a null mutation on the other, the milder missense mutation determined the clinical phenotype.

Mutation Localization Within the Thiolase Protein in Homology to the Yeast Model

Amino acids 6-435 of the human mitochondrial long-chain 3-ketoacyl-CoA thiolase share 31% identity (133 of 429 residues) with the yeast peroxisomal thiolase (Fig. 2). Sequence similarity (66 of 429 residues, 15%) is also extensive. In the yeast protein, C125, H375, and C403 are essential catalytic residues [Mathieu et al., 1997]. The alignment of human and yeast thiolase amino acid sequences demonstrates that these three catalytic residues are conserved in the human thiolase (Fig. 2) and can be assigned to positions C105, H395, and C425. None of the β-subunit mutations we delineated were located within the catalytic domains. However, all missense mutations were located in conserved regions of the thiolase protein. Two premature termination mutations (S143X, R170X) were located in nonconserved regions.

The peroxisomal yeast thiolase has been characterized by crystallography [Mathieu et al., 1997] and is a dimer with two α-helices completely buried in the dimer and sandwiched between two β-sheets (Fig. 3). Based upon the extensive sequence homology and similarity shared by human and yeast thiolase, we suggest that the yeast thiolase crystal structure can serve as a model to align human mutations and predict effects of these mutations on structure and function (Fig. 3). Human missense mutations at positions 84, 88, 143, 196, 198, 209, and 214 are located within α-helices. Mutations at positions 26, 28, and 100 are located within β-sheets. Mutations at positions 170, 247, 261, and 268 are located in random coils of undefined secondary structure (Fig. 3). Mutations at positions 26, 28, 84, 88, and 100 are located in the dimerization domains. Mutations at positions 209, 214, 247, 261, and 268 are located on outer loops of the thiolase protein (Table 3). Thus, we distinguish three structural groups of mutations: missense mutations within or in close proximity to the dimerization domain, missense mutations lying within outer loops, and mutations due to a premature termination or a frameshift that

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Y  21 ESKRKNSLLEKRPEDVVIVAANRSAIGKGFKGAFKDVNTDYLLYNFLNEFIGRFPEPLRA  80
         ++K K +L +     +VV+V   R+         ++KD+    L    L   + R   P
H   6 QTKTKKTLAKPNIRNVVVVDGVRTPFLLSGT-SYKDLMPHDLARAALTGLLHRTSVPKE-  63

Y  81 DLNLIEEVACGNVLNVGAGATEHRAACLASGIPYSTPFVALNRQCSSGLTAVNDIANKIK 140
            +++ +  G V+     +   R A L +G    TP   +    C  S   A+      I
H  64 ---VVDYIIFGTVIQEVKTSNVAREAALGAGFSDKTPAHTVTMACISANQAMTTGVGLIA 120

Y 141 VGQIDIGLALGVESMT-----------------NNYKNVNP-LGMIS-------SEELQK 175
         GQ D+ +A GVE M+                  N  K++   L +IS       + EL
H 121 SGQCDVIVAGGVELMSDVPIRHSRKMRKLMLDLNKAKSMGQRLSLISKFRFNFLAPELPA 180

Y 176 NREAKKCLIPMGITNENVAANFKISRKDQDEFAANSYQKAYKAKNEGLFEDEILPIKLPD 235
           E       MG + + +AA F +SR +QDE+A  S+  A KA++EGL  D ++P  K+P
H 181 VSEFSTSET-MGHSADRLAAAFAVSRLEQDEYALRSHSLAKKAQDEGLLSD-VVPFKVPG 238

Y 236 GSICQSDEGPRPNVTAESLSSIRPAFIKDRGTTTAGNASQVSDGVAGVLLARRSVANQLN 295
               D G RP+ + E ++ ++PAFIK  GT TA N+S ++DG + +L+     A  +
H 239 KDTVTKDNGIRPS-SLEQMAKLKPAFIKPYGTVTAANSSFLTDGASAMLIMAEEKALAMG 297

Y 296 LPVLGRYIDFQTVGVPP-EIMGVGPAYAIPKVLEATGLQVQDIDIFEINEAFAAQALYCI 354
                DF  V   P + + +GP YA PKVLE  GL + DID FE +EAF+ Q L
H 298 YKPKAYLRDFMYVSQDPKDQLLLGPTYATPKVLEKAGLTMNDIDAFEFHEAFSGQILANF 357

Y 355 HKLGID-----------------LNKVNPRGGAIALGHPLGCTGARQVATILRELKKD-- 395
         +  D                 L K N  GG+++LGHP G TG R V      L+K+
H 358 KAMDSDWFAENYMGRKTKVGLPPLEKFNNWGGSLSLGHPFGATGCRLVMAAANRLRKEGG 417

Y 396 QIGVVSMCIGTGMGAAAI                                          413
      Q G+V+ C    G G A I
H 418 QYGLVAACAAGGQGHAMI                                          435
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FIGURE 2. **Alignment of yeast and human thiolase amino acid sequences. The sequences are given using the single letter amino acid code. Dashes (—) designate gaps introduced into the sequences to maximize alignment. Identical residues shared by the yeast (Y) and human (H) proteins are designated by the single letter code shown between the sequences. Similarities of amino acids are designated by the "+" sign between the sequences. The blue coloring shows the three catalytic residues. The green color designates mutation sites present in our patients. The turquoise color shows mutation sites reported in literature [Orii et al., 1997]. The red color defines a single missense mutation reported in literature [Ushikubo et al., 1996].**

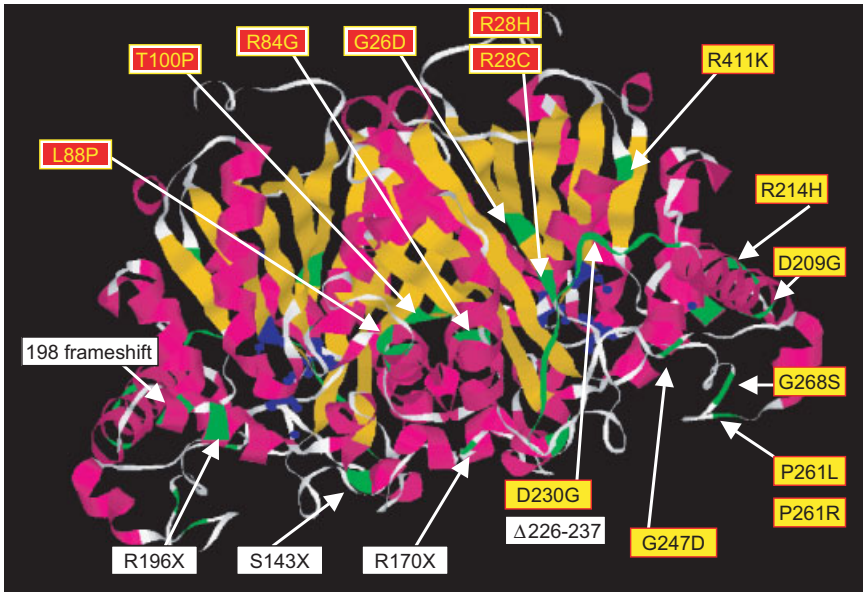

FIGURE 3. **Crystal structure of the 3-ketoacyl-CoA thiolase of *Saccharomyces cerevisiae* and localization of β-subunit mutations. The peroxisomal yeast thiolase has been characterized by crystallography [Mathieu et al., 1997] and is a dimer. All β-subunit mutations, including those reported in literature (R411K, D230G, 226-237del), and their localization within the thiolase protein are shown, as defined by homology and alignment between human and yeast thiolase protein primary structure shown in Figure 2. The mutation localizations are marked green; catalytic residues are blue; α-helices are shown in red; and β-sheets are shown in yellow. Catalytic residues are also displayed in the ball and stick mode. Loop mutations are highlighted in yellow; mutations within the dimerization domain are highlighted in red; and null mutations due to a premature termination, a frameshift, or a deletion are shown in white.**

would likely result in no expression (i.e., be null mutations) because of nonsense-mediated mRNA decay [Perlick et al., 1996].

DISCUSSION

We here define the molecular genetic basis and describe the heterogeneous phenotypes of TFP deficiency due to β-subunit mutations in 15 patients from 13 families (the largest series of patients ever reported). Only four patients with TFP deficiency and underlying β-subunit mutations have previously been described [Ushikubo et al., 1996; Orii et al., 1997]. As in other long-chain fatty acid oxidation defects such as very-long-chain acyl-CoA dehydrogenase (VLCAD) deficiency that catalyzes the first step in the β-oxidation spiral with long-chain fatty acids [Gregersen et al., 2001, Andresen et al., 1999], three phenotypes with mainly cardiac, hepatic, or neuromyopathic involvement can be distinguished. In contrast to previous reports of TFP deficiency secondary to α-subunit mutations, the largest group of our patients with β-subunit mutations is comprised of individuals with late-onset progressive peripheral neuropathy and episodic myoglobinuria. We emphasize that this myopathic phenotype is relatively common in TFP deficiency and compatible with long-term survival, both novel concepts in this FAO disorder.

Overall, the heterogeneous phenotypes in patients with TFP deficiency due to either α- or β-subunit mutations are similar [Hintz et al., 2002; Ibdah et al., 1998; Schaefer et al., 1996]. Because both subunits and their association are essential for stabilizing the trifunctional protein complex [Ushikubo et al., 1996], this observation may not be surprising.

In general, TFP deficiency as in VLCADD, physiologic stressors such as infection, decreased oral intake, or exercise often induce clinical symptoms. In VLCADD, symptoms are preventable and reversible if precipitating factors such as fasting or long-chain fat intake are avoided [Cox et al., 1998; Brown-Harrison et al., 1996]. A relatively normal life can be achieved for years with these preventive measures. Although our four patients with the severe cardiac phenotype of TFP deficiency died within the neonatal period despite immediate intensive treatment, patients with the later-onset myopathic phenotype improved with treatment.

Maternal liver diseases such as HELLP syndrome and acute fatty liver of pregnancy (AFLP) have been associated with pregnancies carrying a fetus affected by LCHADD with an incidence of 19% [den Boer et al., 2002] to 79% [Ibdah et al., 1999; Yang et al., 2002]. With an incidence of 15% in our study and a total of seven cases among 28 reported patients with TFP deficiency (25%) [Isaacs et al., 1996; Chakrapani et al., 2000; our patients], we conclude that maternal liver disease occurs in 15 to 25% of pregnancies with TFP-deficient fetuses due to either α- or β-subunit mutations. We also conclude that this occurrence is not dependent on the severity of the fetal phenotype.

Human trifunctional protein is a hetero-octamer containing four α- and four β-subunits. Mutations in these TFP genes result in two different biochemical phenotypes: isolated LCHAD deficiency and general TFP deficiency. Isolated LCHADD is consistent with relatively normal expression of both subunits and is defined by reduced LCHAD activity. The common α-subunit mutation, E474Q, is the underlying mutation in about 60% [Ibdah et al., 1999; Yang et al., 2002] to 86% [den Boer et al., 2002] of abnormal alleles. This mutation is located within the LCHAD catalytic region and has modest effects on the association of both subunits and the stability of the trifunctional protein. General TFP deficiency is characterized by the loss of both subunits and reduced activity of all three TFP enzymes [Ushikubo et al., 1996; Orii et al., 1997]. Western blot analysis of the β-subunit protein in all patients tested in this study revealed (consistent with the enzyme data) a significant reduction of thiolase protein expression, especially in patients with the lethal phenotype. Mice that completely lack both TFP subunits die within the neonatal period (secondary to hypoglycemia, cardiac dysfunction, and respiratory insufficiency) similar to our four patients with severe TFP deficiency [Ibdah et al., 2001].

Overall, even though the majority are missense mutations, the β-subunit mutations we have delineated alter thiolase and TFP stability and structure sufficiently to severely reduce activities of both subunits and thiolase antigen expression in all patients tested. The extent of reduction in enzyme activity in fibroblasts clearly demonstrated deficiency but did not correlate with the severity of the clinical phenotype. Enzyme activity studies in highly oxidative tissues, such as heart or skeletal muscle, may be needed to correlate the enzymatic defect with the clinical phenotype.

The results from our molecular genetic analysis of the β-subunit prove that TFP deficiency is characterized by molecular heterogeneity. Because six of 13 unrelated patients shared missense mutations at positions 176 or 181-182 on exon 4, we speculate that this region of exon 4 might be a mutational hot spot for β-subunit mutations. α-subunit mutations resulting in TFP deficiency are also molecularly heterogeneous and are not confined to a certain domain of the protein [Brackett et al., 1995; Ibdah et al., 1998; Ibdah et al., 1999; Matern et al., 1999; Hintz et al., 2002].

The majority of patients with β-subunit mutations were compound heterozygotes. Premature termination (null) mutations and alterations of residue 28 were present in the four patients with neonatal death. This

suggests that arginine-28, which lies in the dimerization domain, is critical for protein function or stability. Among individuals with nonresidue 28 missense mutations on one allele and a null mutation on the other (such as the frameshift mutation in Patient 5 or the premature termination mutations in Patients 10 and 11), the milder missense mutations (e.g., R84G, R214H, P261L, P261R) appear to determine the clinical phenotype. In patients with only one known mutation (Patients 2–4, 12, and 14), it is uncertain if the delineated mutation is the one determining the phenotype. Although our results do not allow strict correlation between mutations and any phenotype, particular organ involvement, or death, the occurrence of at least one missense mutation other than the residue-28 missense mutation in all patients with the milder later-onset phenotype is consistent. Therefore, it seems likely that mild missense mutations on at least one allele allow sufficient long-chain fatty acid oxidation under most circumstances and are compatible with long-term survival. This is similar to the genotype–phenotype correlation suggested for VLCAD deficiency [Andresen et al., 1999].

The amino acid homology shared by the yeast and the human thiolase proteins, with conservation of all three catalytic residues (Fig. 2), suggests that structural features are likely conserved and that the known yeast thiolase structure can serve as a model to determine mutation sites. None of the mutations described in our study was located in the catalytic domains. However, all missense mutations were located in conserved regions of the thiolase protein (Figs. 2 and 3). Premature termination mutations usually result in no mRNA expression due to nonsense mediated mRNA decay, so the location of premature termination mutations in the structural model is irrelevant. We speculate that missense mutations in outer loops of the protein (positions 209, 214, 247, 261, 268) do not alter the structure of the protein as much as mutations in regions that are part of the dimerization domains (positions 84, 88, 100). This likely results in an increased rate of degradation, secondary to protein instability. In fact, mutations in outer loops were only found among patients with milder phenotypes. Outer loop mutations may cause hetero-octamer instability because of impaired interactions with other TFP subunits and result in a moderate reduction of thiolase protein expression. This is consistent with our Western blot results.

Missense mutations are expected to present with milder phenotypes than premature termination or frameshift mutations. However, one patient with a homozygous missense mutation presented with the lethal phenotype. Therefore, the mutation site (arginine-28) and its effect on the protein structure must play a major role. We conclude that determination of mutation sites assists in understanding the mechanism by which these mutations affect the protein structure and cause disease.

This study reports the largest series of β-subunit mutations and TFP deficiency, allowing clinical and molecular characterization of this recently discovered disease. In summary, TFP deficiency due to β-subunit mutations is heterogeneous at both the molecular and phenotypic levels and has the presence of mild and severe forms in common with other long-chain fatty acid oxidation defects. The mild myopathic phenotype is the most common presentation. This is consistent with the predominance of missense mutations.

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