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Clear Correlation of Genotype with Disease Phenotype in Very–Long-Chain Acyl-CoA Dehydrogenase Deficiency

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Summary

Very–long-chain acyl-CoA dehydrogenase (VLCAD) catalyzes the initial rate-limiting step in mitochondrial fatty acid β-oxidation. VLCAD deficiency is clinically heterogenous, with three major phenotypes: a severe childhood form, with early onset, high mortality, and high incidence of cardiomyopathy; a milder childhood form, with later onset, usually with hypoketotic hypoglycemia as the main presenting feature, low mortality, and rare cardiomyopathy; and an adult form, with isolated skeletal muscle involvement, rhabdomyolysis, and myoglobinuria, usually triggered by exercise or fasting. To examine whether these different phenotypes are due to differences in the VLCAD genotype, we investigated 58 different mutations in 55 unrelated patients representing all known clinical phenotypes and correlated the mutation type with the clinical phenotype. Our results show a clear relationship between the nature of the mutation and the severity of disease. Patients with the severe childhood phenotype have mutations that result in no residual enzyme activity, whereas patients with the milder childhood and adult phenotypes have mutations that may result in residual enzyme activity. This clear genotype-phenotype relationship is in sharp contrast to what has been observed in medium-chain acyl-CoA dehydrogenase deficiency, in which no correlation between genotype and phenotype can be established.

Introduction

Oxidation of fatty acids in the mitochondria provides the main source of energy in heart and skeletal muscle. Moreover, during periods of fasting, ketone bodies produced by mitochondrial fatty acid oxidation in the liver become the major source of the body's energy needs. Not surprisingly, inherited defects in any of the individual steps of mitochondrial β-oxidation can be expected to result in significant clinical disease. So far, as many as 16 inherited defects affecting the β-oxidation of straight-chain fatty acids have been described (Roe and Coates 1995).

Very–long-chain acyl-CoA dehydrogenase (VLCAD) is one of four acyl-CoA dehydrogenases, with different chain-length specificity, that catalyze the initial rate-limiting step in mitochondrial β-oxidation of fatty acids (Beinert 1963; Aoyama et al. 1995*b*). Patients with defects in short-chain acyl-CoA dehydrogenase (SCAD) and medium-chain acyl-CoA dehydrogenase (MCAD) have been known of for many years (Kølvraa et al. 1982; Amendt et al. 1987). The first patients with VLCAD deficiency were identified as early as 1985 (Hale et al. 1985), but, because the VLCAD enzyme had not yet been identified, these patients were believed to have long-chain acyl-CoA dehydrogenase (LCAD) deficiency. In 1993, two groups of investigators characterized for the first time the enzyme defect in patients with VLCAD deficiency (Aoyama et al. 1993; Bertrand et al. 1993), and more recently, it has been demonstrated that patients previously believed to have LCAD deficiency have VLCAD deficiency instead (Aoyama et al. 1995*b*; Largillière et al. 1995). On the basis of clinical data from seven VLCAD patients, Aoyama and coworkers (1995*b*) suggested that VLCAD deficiency may give rise to more-severe disease than the other β-oxidation defects. Ac-

cording to these investigators, VLCAD deficiency is characterized by disease onset in the first few months of life, frequently in the neonatal period; a high mortality rate (75%); and cardiomyopathy in the majority of patients. In contrast to this, the patients studied by our group (Bertrand et al. 1993; Andresen et al. 1996*a*) generally had a milder phenotype. Recently, Vianey-Saban and coworkers (1998) reported the results of a study of 27 families with established VLCAD deficiency. They reported that the childhood patients could be grouped into two major clinical phenotypes: a severe childhood form, similar to that in the patients described by Aoyama and coworkers (1995*b*), and a milder phenotype, with delayed disease onset, a lower mortality rate, absence of cardiomyopathy, and hypoketotic hypoglycemia as the main presenting feature. In addition, it is becoming evident that a third phenotypic form of the disease exists, namely, a muscular form that presents after childhood exclusively as muscle disease leading to rhabdomyolysis and myoglobinuria, which is often exercise induced and progressive (Ogilvie et al. 1994; Minetti et al. 1998; Smelt et al. 1998).

The human VLCAD cDNA (Aoyama et al. 1995*a*; Andresen et al. 1996*a*) and genomic sequence (Strauss et al. 1995) were identified only a few years ago, and consequently only a small number of patients have been studied at the molecular level. Despite this, 17 different pathogenic mutations have been identified (Aoyama et al. 1995*a*; Strauss et al. 1995; Andresen et al. 1996*a*, 1996*b*; Souri et al. 1996, 1998*a*, 1998*b*; Smelt et al. 1998), indicating that the mutational spectrum in VLCAD deficiency is diverse. In view of the variable clinical phenotypes in this disease, it is tempting to speculate that they could be explained by different mutations in the VLCAD gene that result in different levels of residual enzyme activity. In other inherited diseases—for example, phenylketonuria—it has been demonstrated that such a correlation between mutation type and disease phenotype can be made (Desviat et al. 1997; Kayaalp et al. 1997). The relationship between genotype and phenotype has also been examined in two defects of fatty acid oxidation. In MCAD deficiency there is no clear-cut genotype-phenotype relationship (Andresen et al. 1997). However, in carnitine palmitoyltransferase (CPT II) deficiency, which, like VLCAD deficiency, affects the metabolism of long-chain fatty acids, some correlation between genotype and phenotype has been reported (Taroni et al. 1993; Verderio et al. 1995; Bonnefont et al. 1996; McGarry and Brown 1997).

In the present study we have investigated and characterized the mutations responsible for the VLCAD deficiency in 55 unrelated patients representing all the known clinical phenotypes of the disease and have correlated the mutation types with the clinical phenotypes.

Material and Methods

Patients

We studied 55 families in which the index case had experienced clinical symptoms of VLCAD deficiency (Ogilvie et al. 1994; Roe and Coates 1995; Smelt et al. 1998). In 44 of the families included in the study, the enzyme defect was demonstrated in cultured skin fibroblasts from the index patient by measurement of dehydrogenation of palmitoyl-CoA, with electron transfer flavoprotein (ETF) used as the electron-acceptor (Frerman and Goodman 1985). The enzyme activity of matrix and membrane fractions with palmitoyl-CoA as substrate was assayed in triplicate as described elsewhere (Bertrand et al. 1993; Vianey-Saban et al. 1998). In 14 patients who were not tested by the ETF assay, the enzyme defect was inferred from measurement of the β-oxidation activity in intact cultured fibroblasts with [9,10^{3}H]myristate, [9,10-^{3}H]palmitate, and [9,10-^{3}H]oleate used as substrates, as described elsewhere (Manning et al. 1990; Olpin et al. 1997). In all patients investigated, the enzyme defect was also indicated by one or more of the following: an abnormal acylcarnitine profile in plasma measured by tandem mass spectrometry (Millington et al. 1992), dicarboxylic aciduria without glycine conjugates measured by gas chromatography/mass spectrometry (GC/MS), analysis of urine, identification and quantitation of intermediates of unsaturated fatty acid metabolism in plasma by GC/MS analysis (Onkenhout et al. 1995; Costa et al. 1997), and oxidation of ^{14}C-labeled palmitic acid in intact fibroblasts (Vianey-Saban et al. 1998). To define the disease phenotypes, we collected data on the clinical and biochemical findings in all investigated patients apart from one patient for whom the data were insufficient for categorization. The clinical and biochemical characteristics of the patients are summarized in table 1. The case histories of 12 of the patients included in this study have been published previously (Bertrand et al. 1993; Ogilvie et al. 1994; Onkenhout et al. 1995; Andresen et al. 1996*a*; Brown-Harrison et al. 1996; Gillett et al. 1996; Merinero et al. 1996; Nada et al. 1996; Costa et al. 1998; Smelt et al. 1998). Moreover, patients from 14 of the families included in the present study were studied clinically and biochemically by Vianey-Saban et al. (1998).

Preparation of DNA

Genomic DNA was isolated from cultured fibroblasts or blood samples according to standard methods (Gustafson et al. 1987). DNA was liberated from blood spots and cultured cells as described elsewhere (Gregersen et al. 1991*b*; Andresen et al. 1992).

Table 1

Clinical Classification of Patients and Correlation of Genotype to Phenotype

		PERCENT WITH PHENOTYPE			PERCENT WITH GENOTYPE	
VARIABLE	TOTAL ($n = 54$)	Severe Childhood ($n = 25$ [46%])	Mild Childhood ($n = 21$ [39%])	Adult Onset ($n = 8$ [15%])	Two Null Mutations[a] ($n = 15$ [28%])	Two Missense Mutations/Single– Amino Acid Deletion ($n = 18$ [33%])
Age at onset:						
<3 d	48	76	33	0	80	44
1–11 mo	22	24	29	0	13	17
1–4 years	15	0	38	0	7	11
>13 years	15	0	0	100	0	28
Index patient dead	37	80	0	0	73	17
Dead sibs older than index patient	33	56	19	0	67	33
Families with dead children	50	92	19	0	93	39
>1 episode in first 2 years of life or died in first episode	57	100	29	0	87	44
Cardiomyopathy	52	92	19	13	73	24
Hepatomegaly	61	80	62	0	80	29
Rhabdomyolysis/myoglobinuria	20	0	14	100	0	33
Hypotonia	50	52	62	13	40	29
Hypoketotic hypoglycemia without cardiomyopathy as main presenting symptom	30	0	76	0	7	24

NOTE.—Data are percentages indicating the proportion of patients who have the feature in question.

[a] The R573W and delK258 mutations were categorized as null mutations on the basis of expression data from Souri et al. (1996).

PCR Amplification and Direct Sequencing of the Entire VLCAD Protein-Coding Region

PCR amplifications of all exons, including part of the flanking intron sequences of the human VLCAD gene, were performed with both M13 forward- (-21) and M13 reverse-tagged intron-located primers under standard conditions in an automated thermal cycler (thermal cycler 480, Perkin-Elmer). Primer sequences are available on request (from B.S.A.). The PCR products were subjected to direct bidirectional cycle sequencing by means of DNA dye primer sequencing kits (Prism, *Taq*FS, and BigDye, Perkin-Elmer) in an ABI Catalyst 800 Molecular Biology LabStation (Applied Biosystems). Sequence reactions were performed on semiautomated ABI 373A and ABI 377 sequencers (Applied Biosystems).

Extraction of Total RNA, Northern Blot Analysis, cDNA Synthesis, PCR Amplification of VLCAD cDNA, and Cloning of Amplified VLCAD cDNA

Extraction of total RNA from patient and control fibroblasts was performed by means of an RNAzol kit (WAK-Chemie). Northern blot analysis of 15 μg of total RNA from patients and controls was performed, as described elsewhere, with the VLCAD probe (Andresen et al. 1996a)—a probe specific for intron 10 of the VLCAD gene—and a β-actin probe (Clontech). Four nylon filters containing similar amounts of poly-(A)+ mRNA from different human tissues (MTN-blot I, Clontech) were also used for northern hybridization. To ensure that the observed results are representative, we obtained filters from different batches.

First-strand cDNA synthesis and PCR amplification of VLCAD cDNAs were performed as described elsewhere (Andresen et al. 1996a). PCR products were analyzed by agarose gel electrophoresis. Fragments were cut out of the gel, purified (Qiagen gel extraction kit), and either subjected to direct sequencing or cloned into a pCRII vector (InVitrogen) or a PCRScript vector (Stratagene).

Expression of Wild-Type and Mutant VLCAD in COS-7 Cells

A fragment comprising the entire coding region of human VLCAD cDNA, from a position 42 bp upstream of the ATG start codon to cDNA position 2046, was amplified under standard conditions by means of Turbo Pfu polymerase (Stratagene) from liver cDNA, with a sense primer introducing an *Eco*RI site and an antisense primer introducing an *Xba*I site. This fragment was digested with *Eco*RI and *Xba*I and cloned into the polylinker of the expression vector pCDNA 3.1⁺ (InVitrogen). Resulting clones were sequenced, and one clone (HVLCp9), which harbored a sequence error in the 5′ end of the VLCAD cDNA insert, was chosen for further cloning. A fragment spanning the interval from the *Nhe*I site in the polylinker to the *Hin*dIII site in the HVLCAD cDNA sequence of HVLCp9 was replaced

with a fragment spanning the interval from the *Spe*I site in the polylinker to the internal *Hin*dIII from a PCR error-free 5′ RACE clone characterized previously (Andresen et al. 1996*a*). The resulting plasmid (named "pHVLC-WT") was sequenced, and no PCR errors were identified. To introduce the V243A and T220M mutations into pHVLC-WT, HVLCAD cDNA from patients harboring these mutations was amplified with Turbo Pfu and digested with *Kpn*I and *Hin*dIII, and the resulting fragment was purified and cloned back into the plasmid pHVLC-WT, replacing the corresponding fragment of the wild-type sequence. To confirm that only the mutations, and no PCR-derived errors, were present in the exchanged fragments, we sequenced the constructed plasmids, named "pHVLC-V243A" and "pHVLC-T220M."

Transfection of *COS-7* cells was performed according to a standard calcium phosphate coprecipitation method, and expression of VLCAD protein was assayed 36 h posttransfection from cells transfected with pHVLC-WT, pHVLC-V243A, pHVLC-T220M, and pCDNA 3.1$^+$, respectively. The conditions for growth and transfection of cells have been described elsewhere (Jensen et al. 1992).

Aliquots containing ~25 μg of total protein from the cleared supernatant of lysates from the transfected *COS-7* cells were tested by western blot analysis, as described below. Measurement of VLCAD activity was by ferricenium assay that used palmitoyl-CoA as substrate (Lehman et al. 1990; Scholte et al. 1992). All transfections were performed at least twice, and enzyme activity was measured in duplicate.

Western Blot Analysis

Frozen cell pellets from transfected *COS-7* cells and cultured fibroblasts from patients and controls were lysed in 100 μl of lysis buffer (50 mM Tris-HCL at 7.7 pH, 5 mM EDTA at 7.4 pH, 1% Triton X-100, 250 mM sucrose, 1 mM DTT, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride). After 1 μl of Benzonase was added, the lysates were incubated at 37°C for 1 h before centrifugation at 10,000 *g* for 3 min. The cleared lysates were transferred to new vials, and the protein concentration was determined with a modified Bradford assay kit (BioRad). SDS-polyacrylamide gel electrophoresis and western blot analysis with our VLCAD antibody (Andresen et al. 1996*a*) and an antibody directed against ETF as a control were performed as described elsewhere (Andresen et al. 1996*a*).

LCAD Mutation–Specific Assay

We designed a PCR/restriction enzyme cleavage–based mutation-specific assay for the 997A→C mutation in LCAD cDNA (Yamaguchi et al. 1993). The assay is based on the principle of PCR-based introduction of diagnostic and control restriction enzyme sites for the restriction enzyme *Mbo*I. Primer sequences are available on request (from B.S.A.). The assay was carried out as described, elsewhere, for similar assays (Gregersen et al. 1991*b;* Andresen et al. 1992, 1997), with genomic DNA used as the template for the PCR.

Identification and Characterization of the 463-bp Sequence Upstream from the ATG Translation-Initiation Codon of the Human VLCAD Gene

Adaptor-ligated genomic DNAs from a Genome-Walker kit (Human GenomeWalker kit, Clontech) were amplified as recommended by the supplier with the adaptor primers AP1 (first amplification) and AP2 (second amplification) and the gene-specific primers −44AS (first amplification) and −55AS (second amplification) (Human GenomeWalker kit, Clontech). Information about primers is available on request (from B.S.A.). In one of the libraries (EcoRV) we obtained a product of ~700 bp. By direct sequencing of this product, we obtained 463 bp of sequence upstream from the ATG start codon. A fragment spanning the identified 463-bp sequence was amplified and sequenced from DNA from VLCAD patients and Danish controls, under standard conditions.

Results

Clinical Presentation

The clinical and biochemical findings in our patients are summarized in table 1. The data show a broad clinical spectrum of disease, with all the known phenotypes being presented—two childhood forms and one adult form (Vianey-Saban et al. 1998). As illustrated in the table, the severe childhood form is characterized by early onset of symptoms, usually in the neonatal period, and a high mortality rate or recurrent disease episodes, with low tolerance of fasting. Cardiomyopathy is frequently observed in this group of patients. The milder childhood form (table 1) is characterized by delayed onset of symptoms with low mortality and often occurrence of only a single episode or a few mild episodes of metabolic decompensation. Cardiomyopathy is rare, and the main clinical feature at presentation is hypoketotic hypoglycemia. Hepatomegaly and hypotonia are frequently observed in both childhood forms. The adult form of the disease (table 1) is characterized by onset of disease after childhood, isolated skeletal muscle involvement with recurrent episodes of muscle pain, rhabdomyolysis, and myoglobinuria, usually triggered by exercise or fasting (Ogilvie et al. 1994; Minetti et al. 1998; Smelt et al. 1998; Straussberg et al. 1997). The 55 patients manifested the three phenotypes as follows: 25, severe child-

hood; 21, milder childhood; and 8, adult onset. One patient could not be categorized because of insufficient clinical and biochemical data. Residual VLCAD enzyme activities did not differ significantly in cultured fibroblasts from patients with the different clinical phenotypes. This has been observed previously (Vianey-Saban et al. 1998) and probably occurs because the assays are undertaken for diagnostic purposes rather than for comparison of residual enzyme activities. The fact that our patients are of many different ethnic origins (there are patients from 19 different countries located in North and South America, Australia, Europe, Asia, and Africa) demonstrates that VLCAD deficiency is not primarily a disease affecting white people, as is observed with MCAD deficiency (Tanaka et al. 1997).

Sequence Analysis by Direct Sequencing of the Entire VLCAD Protein-Coding Region

We analyzed 55 index patients by sequencing the entire VLCAD protein-coding region, and available family members from 21 of the families were tested for the relevant mutations (36 different mutations) by sequence analysis. In table 2, the mutations identified are listed along with the number of patients harboring them. Our results show that the mutational spectrum is very wide, with 58 different mutations—only 18 of which are present in >1 patient—identified in 110 mutant alleles from 55 unrelated patients. In 47 families we identified mutations in both VLCAD alleles from the index patient, but in 8 families we could identify the mutation in only one of the two alleles. In five patients we identified three mutations in their two VLCAD alleles (table 2). The nature of the double mutant alleles could be established when the patient was homozygous (the V243A/V277A allele and the G3D/R429W allele) or when the mutant alleles from the patient were cloned and sequenced (V243A/V277A and delE341/L562I [Andresen et al. 1996a]). Interestingly, the V243A, R429W, and delE341 mutations were also observed without the V277A, G3D, and L562I mutations, respectively, in other patients. The V277A, G3D, and L562I mutations were not observed without the simultaneous presence of the V243A, R429W, and delE341 mutations. This could indicate that the V243A, R429W, and delE341 mutations have arisen more than once, or that the V277A, G3D, and L562I mutations have occurred on alleles already harboring the other mutations.

There were 42 different single base pair substitutions, 14 of which were located in a CpG dinucleotide and could have resulted from the mutational mechanism of deamination of a 5′-methylated C residue (Coulondre et al. 1978). There is evidence that the CpG dinucleotides in the codon for arginine326 and arginine429 are mutational hot spots, since C→T (R326C and R429W) and

G→A (R326H and R429Q) mutations of the CpG dinucleotide are present in both codons. Together with the facts discussed above, this could indicate that the R429W mutation has arisen twice, once in an allele with the G3D mutation and once in an allele without it. The G3D mutation is probably not disease associated, since mouse VLCAD (GenBank/European Molecular Biology Laboratories [EMBL] accession number Z71189) has a glutamate at this position, indicating that a negatively charged residue, such as aspartate, is tolerated at this position.

Thirty-one of the identified mutations changed an amino acid (missense mutations), and three of the mutations resulted in a single–amino acid deletion. All except three (L462P, L562I, and R573W) of the missense and single–amino acid deletion mutations were located in the acyl-CoA dehydrogenase domain, which displays a high similarity to the other acyl-CoA dehydrogenase enzymes (Andresen et al. 1996a). However, only seven of them were located in codons of amino acid residues that are completely conserved.

Twelve of the identified mutations were small deletion/ insertions that altered the reading frame, resulting in introduction of a premature-termination codon (PTC). In addition, three mutations directly created a PTC, and nine mutations affected VLCAD mRNA splicing by changing splice consensus sequences or introducing new 3′ splice sites. All the splice mutations (except the Gg→Cac and +2T→C mutations in intron 10) were examined by northern blot analysis of fibroblast total RNA from patients and/or analysis of PCR-amplified cDNA (see fig. 1 and below). They were all shown to affect splicing severely, resulting in production of misspliced VLCAD mRNAs that were present in severely decreased amounts (table 2). All 15 of the PTC mutant VLCAD mRNAs, as well as the misspliced VLCAD mRNAs (except the −6G→A mutation in intron 18), encode a VLCAD protein that would be missing essential parts of the enzyme, such as the active site glutamate 422 (Souri et al. 1998a), if they were to be translated. Similar to the splice mutations, all examined PTC mutations were shown to have resulted in severely decreased amounts of normal-sized VLCAD mRNA (see table 2 and below). Given this, we believe that all 15 PTC mutations and the 9 splice mutations should result in no residual VLCAD enzyme activity.

Northern Blot Analysis—Intron 10 Retention

We performed northern blot analysis of fibroblast total RNA from 46 of the patients and 4 controls with the VLCAD probe (Andresen et al. 1996a). As shown in figure 1A, two bands with varying intensities were detected in the patients. The presence of a weak band that migrated more slowly than the normal-sized VLCAD

Table 2

Mutations in the VLCAD Gene from 55 Unrelated Patients

Category and Mutation	Location	CpG[a]	Change	MCAD[b]	Protein[c]	mRNA[d]	Alleles/Patients[e]	Nationality of Index Patient	Initial Description of Mutation
Null mutations:									
+1G→A	Intron 1	−	Splice		−	−	1/1	USA	
del102	Exon 1	−	Truncation		−	−	5/3	Netherlands	
del249-50	Exon 4	−	Truncation		−	...	1/1	UK	
del296-97	Exon 5	−	Truncation		−	...	1/1	UK	
398G→A	Exon 6	−	W93X		−	...	1/1	Italy	
433C→T	Exon 6	−	Q105X		...	...	1/1	Greece	
del644-47	Exon 8	−	Truncation		−	−	2/2	UK, USA	
685C→T	Exon 8	−	R189X		−	−	2/2	Netherlands, Norway	
del708-09	Exon 8	−	Truncation		−	−	2/1	Turkey	
del710-11	Exon 8	−	Truncation		...	...	2/1	Lebanon	
−2A→C	Intron 8	−	Splice		−	−	2/2	Austria, Italy	
−11T→G	Intron 8	−	Splice		−	−	1/1	UK	
ins2bp-778	Exon 9	−	Truncation		−	−	1/1	France	
del799-802	Exon 9	−	Truncation		...	−	2/1	Israel	
del881-87	Exon 10	−	Truncation		...	...	1/1	UK	
del888/89	Exon 10	−	Truncation		...	...	1/1	Netherlands	
del890-92	Exon 10	−	delK258[f]	L213	−	+	2/2	Switzerland, Netherlands	Souri et al. 1996
del918-19	Exon 10	−	Truncation		−	...	1/1	Germany	
GG→CAC	Intron 10	−	Splice		−	...	1/1	France	
+2T→C	Intron 10	−	Splice		−	...	1/1	Switzerland	
+1G→A	Intron 11	−	Splice		−	−	2/2	Netherlands, UK	Strauss et al. 1995; Aoyama et al. 1995a
−15A→G	Intron 11	−	Splice		...	−	2/1	UK	
del1245-66	Exon 12	−	Truncation		...	...	1/1	Argentina	
1269G→A	Exon 12	+	S383S[g]	G337	...	−	1/1	Austria	
−6G→A	Intron 17	−	Splice		−	−	5/4	France, Turkey, UK, USA	
1837C→T	Exon 20	+	R573W[f]		...	...	1/1	Norway	Strauss et al. 1995; Souri et al. 1996
Missense mutations/single–amino acid deletions:									
128G→A	Exon 2	+	G3D[h]		...	+	4/2	Morocco	
473C→A	Exon 6	−	T118N	F78	−	+	1/1	Italy	Andresen et al. 1996b
476A→G	Exon 6	−	Q119R	D79	−	+	1/1	France	
520G→A	Exon 7	+	V134M	V95	−	+	1/1	UK	
553G→A	Exon 7	+	G145S	G104	−	+	1/1	Germany	
652G→A	Exon 8	−	E178K	E137	−	+	1/1	Spain	Andresen et al. 1996b
728T→G	Exon 8	−	L203R	I160	−	+	1/1	UK	
740A→C	Exon 8	−	K207T	K164	−	+	1/1	UK	

Nucleotide change	Location	CpG [a]	Amino acid change	MCAD residue [b]	Protein [c]	mRNA [d]	No. of alleles [e]	Countries	Reference
779C→T	Exon 9	+	T220M	F177	+	+	5/5	France, Germany, Italy	
del830-32	Exon 9	−	delK238[i]	A193	−	+	2/2	Netherlands	
842C→A	Exon 9	−	A241D	G196	−	+	1/1	France	
848T→C	Exon 9	−	V243A	I198	−	+	10/9	Argentina, France, Netherlands, UK, USA	Andresen et al. 1996a
869G→A	Exon 9	−	G250D	G205	−	+	1/1	Australia	Andresen et al. 1996a
881G→A	Exon 10	−	G254E	G209	−	+	1/1	Australia	Andresen et al. 1996b
897G→T	Exon 10	−	K259N	N214	−	+	1/1	UK	Andresen et al. 1996b
950T→C	Exon 10	−	V277A[h]	V232	−	+	3/2	UK, USA	Andresen et al. 1996a
1054A→G	Exon 11	−	M312V	A267	−	+	1/1	UK	
1096C→T	Exon 11	+	R326C	R281	+	+	1/1	USA	Andresen et al. 1996a
1097G→A	Exon 11	+	R326H	R281	+	+	2/2	UK	
del1141-43	Exon 11	−	delE341	F296	−	+	2/2	Netherlands, USA	Andresen et al. 1996a
1213G→C	Exon 12	−	D365H	V319	−	+	1/1	Spain	
1322G→A	Exon 13	−	G401D	G355	−	+	4/4	Netherlands, UK, USA	Andresen et al. 1996a
1349G→A	Exon 14	+	R410H[i]	K364	+	+	2/2	Netherlands, Spain	Smelt et al. 1998
1358G→A	Exon 14	+	R413Q[i]	R367	+	+	3/2	Greece, former Yugoslavia	
1360G→A	Exon 14	−	D414N[i]	D368	−	...	1/1	UK	
1367G→A	Exon 14	+	R416H	K370	+	...	1/1	UK	
1375C→T	Exon 14	+	R419W	Q373	+	+	1/1	UK	
1388G→A	Exon 14	−	G423E	G377	−	−	2/2	UK	
1405C→T	Exon 14	+	R429W[i]	R383	+	+	7/4	Lebanon, Morocco, UK	
1406G→A	Exon 14	+	R429Q[i]	R383	+	...	2/1	Turkey	
1505T→C	Exon 15	−	L462P[f]		−	...	1/1	Laos	
1804C→A	Exon 18	−	L562I[h]		−	+	1/1	USA	Andresen et al. 1996a
Total	20 exons		58 different changes				102 alleles	19 countries	

NOTE.—The nature and locations of the mutations identified in 55 investigated index patients are listed. All numbering is according to the VLCAD cDNA sequence by Andresen et al. (1996a) (GenBank/EMBL accession number X86556), except for mutations located in introns, which are numbered relative to the exon-intron boundaries (Strauss et al. 1995) (GenBank/EMBL accession number L46590). The mutations have been grouped according to phenotype severity as "null mutations" and "missense mutations /single–amino acid deletions" as described in the text.

a Plus (+) and minus (−) signs indicate whether the base substitution could result from deamination of a CpG hot spot.

b Residues located at the corresponding positions in mature MCAD.

c Results of western blot analysis of immunoreactive protein derived from patient cells. A plus sign (+) indicates normal-sized protein is present in normal amounts; a minus sign (−) indicates the amount of protein is decreased; an ellipsis (...) indicates the mutation has not been analyzed.

d Results of northern blot analysis of mRNA and PCR analysis of cDNA. A plus sign (+) indicates normal-sized mRNA is present in normal amounts; a minus sign (−) indicates the amount of mRNA is decreased; an ellipsis (...) indicates the mutation has not been analyzed.

e No. of identified alleles with respective mutation/no. of unrelated patients harboring the mutation.

f Mutation tested by Souri et al. (1996) and found not to result in any detectable residual enzyme activity.

g Silent mutation, located in the last nucleotide of exon 12, that affects splicing.

h Mutation identified as a second mutation on an allele harboring another pathogenic mutation, and not included in the total no. of alleles (102).

i Mutation that affects residue where a pathogenic mutation in the homologous residue in another acyl-CoA dehydrogenase has been identified. The references are as follows: delK238: SCAD G185S (Corydon et al. 1996); R410H: glutaryl-CoA dehydrogenase (GCD) R402Q/W; D414N: GCD A421T/V (Goodman et al. 1998); R429Q/W: GCD N406K; R429Q/W: GCD A421T/V (Goodman et al. 1998); and R413Q: SCAD R359C (Gregersen et al. 1998).
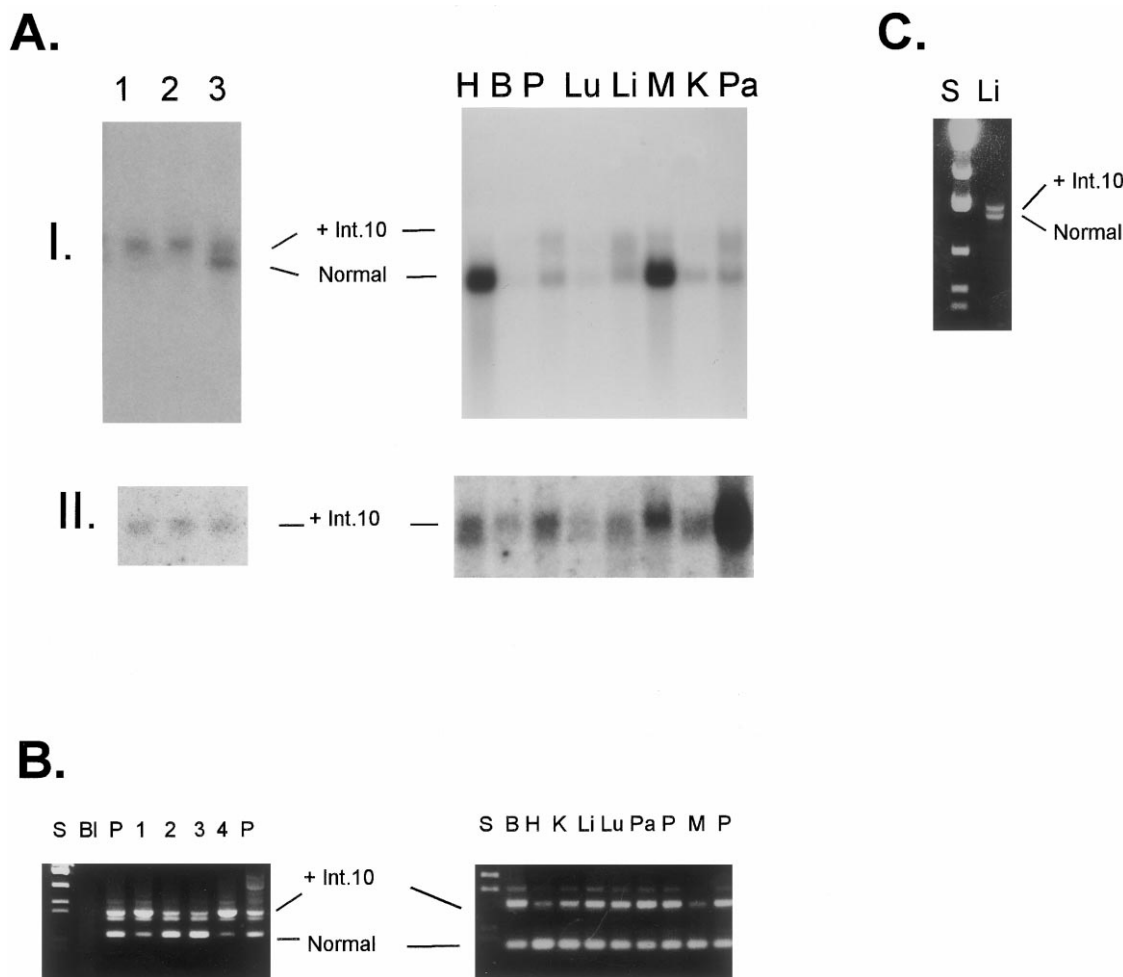
Figure 1   *A,* Northern blot analysis of fibroblast total RNA from three patients (1– 3) and poly(A⁺) mRNA from different human tissues, performed with a full-length VLCAD cDNA probe (*I*) and a VLCAD intron 10–specific probe (*II*). Patients 1 and 2 both have two PTC mutations, and patient 3 has two missense mutations. H = heart tissue, B = brain, P = placenta, LU = lung, LI = liver, M = skeletal muscle, K = kidney, and PA = pancreas). "Normal" indicates normal-sized VLCAD mRNA, and "+INT 10" indicates the position of the VLCAD mRNA with intron 10 retained. *B,*PCR of VLCAD cDNA from four patients and different human tissues, performed with a sense primer located in exon 7 and an antisense primer located in exon 12. Patients 1 and 4 both have two PTC mutations, and patients 2 and 3 both have two missense mutations. S = size marker, and BL = blank amplification with no cDNA added. Other abbreviations are the same as in *A. C,* PCR of full-length VLCAD cDNA from human liver, performed with primers located in exon 1 (sense) and exon 20 (antisense). Abbreviations are the same as in *A* and *B.*

mRNA band has also been observed previously, and it was suggested that it was caused by nonspecific hybridization of the very G/C-rich probe (Andresen et al. 1996*a*). This band is also visible in northern blots produced in other laboratories but has not been discussed (Souri et al. 1996). We observed that in patients with two mutations that result in the introduction of a PTC in the VLCAD mRNA—either directly, because of small insertions/deletions, or indirectly, as a result of missplicing—the normal-sized VLCAD mRNA band was absent, but the slower-migrating band was still present (fig. 1*A*). Because we had, at the same time, observed two bands of equal intensities (fig. 1*C*) when we am-

plified the entire coding region of VLCAD from human liver cDNA, we began to suspect that the slower-migrating band was not caused by nonspecific hybridization but instead represented an alternative form of VLCAD mRNA. Cloning and sequence analysis of the two bands, amplified from human liver cDNA, showed that they had identical sequences, except that the slower-migrating band also contained the entire intron 10 sequence. We then used a probe that exclusively recognizes intron 10 for hybridization of our patient northern blots and four multiple-tissue northern blots. This showed that the slower-migrating band indeed harbors intron 10 and that the normal-sized band was absent when the

blots were hybridized with the intron 10–specific probe. Hybridization of the blots with probes specific for human MCAD and LCAD (Andresen et al. 1996a) produced only a single band of the expected size, indicating that the blots did not harbor significant amounts of unspliced nuclear mRNA.

We also investigated VLCAD cDNA from 41 of our patients and from eight different tissue types from control individuals (human Multiple Tissue cDNA panel) by PCR amplification that used four sets of primers located in different exons flanking intron 10. In figure 1B, the results obtained with one set of primers (sense primer in exon 7 and antisense primer located in exon 12) used in four patients and eight different human control tissues are shown. Two major bands were observed, excised from the gel, and shown by sequence analysis to represent either normal VLCAD cDNA sequence or normal VLCAD cDNA sequence with intron 10 retained. The third weak band that was observed was most probably a heteroduplex band.

As illustrated in the figure, patients with two PTC/splice mutations had very small amounts (nearly none) of the band representing the normal VLCAD mRNA, but the intron 10–containing band was still present and more abundant. In patients with two missense mutations, the band corresponding to normal VLCAD mRNA was the most abundant, and much smaller amounts of the intron 10–containing mRNA were observed. This pattern is consistent with our results from the northern blots and, together with our results from sequence analysis of amplified cDNAs, shows that all of the six PTC mutations and seven splice mutations that could be examined in this way result in severely decreased amounts of normal-sized VLCAD mRNA (table 2).

Sequence analysis of intron 10 showed that it is 267 bp long and that an in-frame stop codon is present. Translation of the intron 10–containing VLCAD mRNA would result in a protein of ~41 kD. This protein would be devoid of enzyme activity, since it lacks important parts of the VLCAD enzyme, such as the active site glutamate 422. The role of intron 10 retention is not clear at present, but one could speculate that it represents some sort of regulation, and it is interesting to note that the tissue-specific distribution of the VLCAD mRNA with intron 10 retention differs from that of the normal-sized VLCAD mRNA, with the highest amounts observed in liver and pancreas tissue.

Although the role of the VLCAD mRNA with intron 10 retained remains obscure, we do know that the intron 10–containing VLCAD mRNA encodes a nonfunctional protein. Therefore, we conclude that patients in whom we observe only the intron 10–containing VLCAD mRNA cannot be expected to produce functional VLCAD enzyme.

Expression of Wild-Type and Mutant VLCAD in *COS-7 Cells*

As an initial approach to test the molecular defect of the missense mutations, we decided to analyze the two most frequently observed missense mutations, V243A (in nine unrelated families) and T220M (in five unrelated families), by overexpression of mutant protein in *COS-7* cells. Measurements of the VLCAD activity in *COS-7* cells showed that cells transfected with the pHVLC-WT plasmid, encoding wild-type VLCAD protein, had an enzyme activity approximately six times higher than the endogenous enzyme activity observed in cells transfected with the pCDNA3.1⁺ vector without VLCAD cDNA inserted. Both cells transfected with the pHVLC-V243A plasmid and those transfected with the pHVLC-T220M plasmid had an enzyme activity above the endogenous level. The V243A and T220M mutant VLCADs had ~20%–25% and ~5% residual enzyme activity, respectively. Western blot analysis (fig. 2) showed that immunoreactive protein with a size corresponding to that of mature VLCAD protein (66 kD) was present both in cells expressing wild-type VLCAD and in cells expressing the mutant proteins V243A and
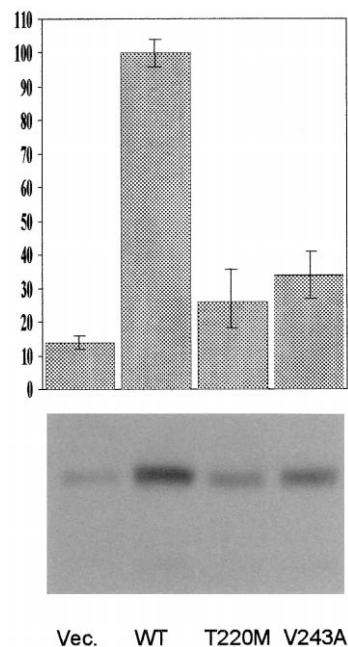

Figure 2   Expression of wild-type and mutant VLCAD in *COS-7* cells. *Upper panel*, Activity measurements are indicated as percentage of wild-type VLCAD. Bars are mean values of duplicate measurements from two separate transfection experiments. Thin lines indicate the maximum and minimum value measured. *Lower panel*, Western blot of *COS-7* cells harboring expression vectors encoding wild-type (WT) and V243A and T220M mutant VLCAD protein, respectively. The control is *COS-7* cells harboring the expression vector pcDNA3.1⁺ with no VLCAD cDNA insert (vector).

T220M. The amounts of immunoreactive protein observed in cells harboring the expression vector without VLCAD cDNA inserted (representing the endogenous VLCAD protein) were much lower than the amounts observed in cells transfected with expression vectors for wild-type or mutant VLCAD. The amounts of immunoreactive protein observed in the transfected cells were proportional to the corresponding enzyme activities (fig. 2), indicating that the mutant proteins have considerable amounts of enzyme activity and that the molecular defect mainly affects the amounts of mutant protein formed.

Western Blot Analysis of Patient Cells

Cultured fibroblasts harboring 43 different mutations, from 37 of the patients, were analyzed by western blot analysis with our VLCAD antibody and an antibody directed against ETF as a control. In figure 3, the results from analysis of four patients and a control are shown. In all analyzed patient tissues, the amounts of immunoreactive VLCAD protein were reduced, although to different degrees, compared with normal controls (table 2). Therefore, we conclude that, in all examined patients, the mutations led to drastically reduced amounts of immunoreactive protein.

Testing for 44 Different Mutations Located in Exons 8–15 in 100 Normal Alleles

Forty-four of the 58 different mutations identified in the patients (located in 54 different alleles) are located in exons 8–15 (table 2). These 44 different mutations represent 82 of the 102 mutant alleles identified in the patients. Thus, we could screen for 80% of all mutant VLCAD alleles by sequencing six amplicons (exons 8, 9, 10, 11, 12-13, and 14-15). To investigate whether the 44 mutations are present in normal controls, we amplified these amplicons in tissue samples from 50 Danish control individuals and sequenced them in one direction. None of the 44 different mutations were observed in any of the 100 alleles tested. This indicates that none of the mutations are polymorphic in a white population such as Danes.

LCAD Variants

We investigated the frequency of the 997A and 997C LCAD variant alleles (Yamaguchi et al. 1993) in Danish control individuals and in our patients, using a mutation-specific assay. Our results (table 3) show that the LCAD variant alleles are polymorphic in the Danish population and that the frequencies of the two variant alleles in our patients do not differ significantly from those of the control population. Moreover, western blot analysis, with an LCAD antibody, of fibroblasts from patients with LCAD 997C/997C, 997A/997C, or 997A/997A genotypes revealed no apparent difference in the
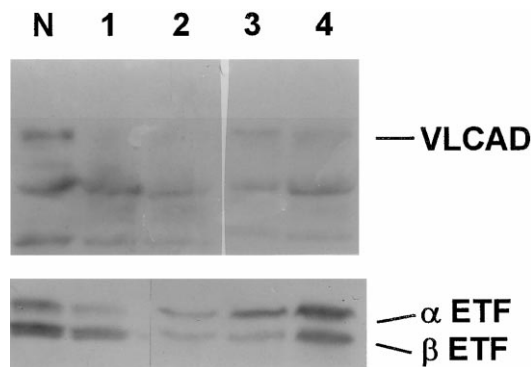

Figure 3 Western blot analysis of total fibroblast protein from a normal control (N) and four VLCAD patients (lanes 1–4), performed with anti-VLCAD and anti-ETF antibodies.

amounts of immunoreactive LCAD protein (results not shown).

Sequence Analysis of the 463-bp Sequence Upstream of the ATG Translation-Initiation Codon of the Human VLCAD Gene in Patients and Controls

To search for mutations located in regulatory sequences, we identified and characterized 463 bp of nucleotide sequence upstream from the ATG translation-initiation codon of the human VLCAD gene. Using our 463-bp upstream sequence to search GenBank (using BLASTn at the National Center for Biotechnology Information), we identified an HTGS sequence (accession number AC003688) from a chromosome 17–derived clone that showed 97% identity in the entire 463-bp overlap. The HTGS sequence showed similar high identity to the nucleotide sequence of the entire VLCAD gene (accession number L46590). This indicates that our sequence indeed represents the 463-bp continuous sequence upstream of the VLCAD translation-initiation codon. Computer analysis (PatSearch 1.1 and MatInspector 2.2, using Transfac 3.4 at the Transcription Element Search System site) and manual analysis identified potential binding sites for several transcription factors, some of which have previously been identified in the promoter region of the human MCAD gene (Zhang et al. 1992). This indicates that the 463-bp upstream sequence may have some regulatory function. This is corroborated by our observation that the 463-bp sequence shows promoter activity when inserted in a luciferase reporter construct (B. S. Andresen, unpublished data).

We then amplified and sequenced the 463-bp fragment in tissue samples from 39 of our patients. The only difference detected was a deletion of a 15-bp perfect repeat sequence corresponding to position −64 to −78 relative to the ATG start codon (Andresen et al. 1996a). This

Table 3

No. of Alleles with Each of the LCAD Variant Alleles

| | | PHENOTYPE[a] | | | |
ALLELE	TOTAL PATIENTS[a]	Mild Childhood	Severe Childhood	Adult Onset	CONTROLS[a]
977A (K333)	68/108 (63)	25/42 (60)	32/48 (67)	9/16 (56)	86/126 (68)
977C (Q333)	40/108 (37)	17/42 (40)	16/48 (33)	7/16 (44)	40/126 (32)

[a] Values in parentheses are percentages.

15-bp deletion was identified in either homozygous or heterozygous form in 23 patients in whom we had already identified mutations in the coding region of both VLCAD alleles. Interestingly, five of six examined patients, in whom we had identified only one mutation in the coding region of one of their VLCAD alleles, were homozygous for the promoter sequence with the normal sequence (GenBank/EMBL accession numbers AJ012053 and X86556). Forty-two percent of patient alleles had the 15-bp deletion and 58% had the normal sequence. Investigation of 58 Danish control individuals showed the 15-bp repeat variation to be polymorphic, with an allele distribution of 43% with the 15-bp deletion and 57% with the normal sequence. Thus, the 15-bp deletion does not seem to be pathogenic by itself but may serve as an allelic marker. The 463-bp sequence has been deposited in the GenBank/EMBL database (accession number AJ012053).

Discussion

It is apparent from the present study and previous studies that VLCAD deficiency is a clinically heterogeneous disease that can be divided into three major disease phenotypes (Ogilvie et al. 1994; Roe and Coates 1995; Aoyama et al. 1995b; Andresen et al. 1996a; Smelt et al. 1998; Vianey-Saban et al. 1998). The aim of this study was to investigate whether these disease phenotypes can be explained by differences in patient genotypes.

Our results confirm that the mutational spectrum in VLCAD deficiency is very wide. In the 55 patients investigated, we identified 58 different mutations, of which 45 have not been reported previously. This means that a total of 62 mutations are now known in the VLCAD gene. In marked contrast to MCAD deficiency and LCHAD deficiency (Gregersen et al. 1991a, 1991b; Yokota et al. 1991; Ijlst et al. 1994), there is no prevalent pathogenic mutation. The pathogenic nature of the mutations identified in our patients was supported by several lines of evidence: The entire protein-coding region was sequenced in all patients, ensuring that the identified mutations were the only mutations present. All of the 44 different mutations that were investigated were excluded in 100 alleles from normal controls. Twenty-four

of the identified mutations were present in more than one patient allele, and, in all 21 families investigated, the identified mutations were located in separate alleles and segregated with the disease. Furthermore, results from northern blot analysis, analysis of VLCAD cDNA, western blot analysis, and overexpression of some of the mutant proteins in eukaryotic cells indicate that the identified mutations are indeed pathogenic.

In addition to our investigation of the protein-coding part of the VLCAD gene, we identified and characterized 463 bp of sequence upstream of the VLCAD translation-initiation codon both in patients and in normal controls. We did not identify any variations in this region, except for a 15-bp deletion that was found to be polymorphic and consequently not by itself pathogenic. Despite this, our results suggest that the 15-bp sequence may have some effect on VLCAD gene expression, rendering individuals susceptible to disease if a pathogenic mutation is present in the other VLCAD allele. It is obvious, however, that further studies are needed to investigate this.

To investigate a possible genotype-phenotype relationship, we categorized the mutations into two classes according to their severity: "null" mutations, which would inevitably lead to no residual enzyme activity, and missense mutations or single–amino acid deletions, which might or might not lead to residual enzyme activity (table 2).

Twenty-four of the mutations in our patients give rise to a protein lacking vital parts of the enzyme because of missplicing and/or direct or indirect introduction of a PTC. These were all categorized as null mutations, since the encoded protein would not be expected to have any residual VLCAD enzyme activity. Furthermore, the presence of a PTC (including those resulting from missplicing) usually dramatically reduces the steady-state amounts of mRNA (Maquat 1995). Consistent with this, all of the PTC mutations that were investigated resulted in severely decreased amounts of normal-sized VLCAD mRNA. Several different mechanisms have been suggested for PTC recognition and degradation of the mRNA. All of them rely on recognition of the PTC by some sort of "mRNA surveillance machinery," followed by degradation of the mutant mRNA either in the nucleus or in the cytosol (Maquat 1995). In this context,

our observation of VLCAD mRNA with intron 10 retained is puzzling. Intron 10 contains an in-frame PTC, and the intron 10–containing VLCAD mRNA should, therefore, be recognized and degraded. Furthermore, the amount of intron 10–containing VLCAD mRNA appears to be unaffected by mutations introducing PTCs into the coding sequence upstream of intron 10. This may indicate that turnover of intron 10–containing VLCAD mRNAs is independent of the translational machinery, since this machinery would recognize the most upstream PTC first. Further studies are clearly needed to resolve this enigma, but there can be no doubt that the identified PTC/splice mutations are null mutations.

The severity of the effects of the 31 identified missense mutations and three single–amino acid deletions is more difficult to evaluate. Our western blot analyses of patient fibroblasts showed that all of the 43 mutations investigated lead to decreased steady-state amounts of VLCAD protein (table 2). This finding, together with the fact that as many as six of the identified mutations are located in residues where mutation of the corresponding residue in at least one other acyl-CoA dehydrogenase has been shown to cause disease (table 2), may suggest that the molecular defect of the VLCAD mutations is similar to that of mutations causing other acyl-CoA dehydrogenase defects. In MCAD and SCAD deficiencies, the majority of mutations cause compromised folding or assembly of the acyl-CoA dehydrogenase protein, resulting in decreased amounts of functional enzyme and varying degrees of residual enzyme activity (Andresen et al. 1993, 1994, 1997; Bross et al. 1993, 1998; Gregersen et al. 1998). We observed considerable levels of residual enzyme activity in the V243A mutant protein and residual enzyme activity above background in the T220M mutant protein when we investigated them by overexpression in *COS-7* cells. This demonstrates that the two most frequent missense mutations in VLCAD have a molecular-defect mechanism that resembles that previously observed for the other acyl-CoA dehydrogenases.

Although the various missense mutations and single–amino acid deletions will result in different levels of residual enzyme activity, we classified all of them (except delK258 and R573W, which have both been demonstrated by Souri et al. [1996] to have no residual enzyme activity) as resulting in milder phenotypes than the null mutations. Obviously, this is an oversimplification, but nevertheless, we were able to observe a correlation between genotype and phenotype in the investigated families. In the severe childhood disease phenotype, the majority (71%) of identified alleles were of the null type, whereas in the milder childhood and the adult disease phenotypes, the majority of alleles (82% and 93%, respectively) harbored mutations that might result in residual enzyme activity.

To make the correlation more precise, we excluded patients who had one mutation of each type and compared only the phenotypes of patients who had the same type of mutation in both alleles (i.e., two null mutations or two missense or single–amino acid deletion mutations) (table 1). Fourteen of the patients with two null mutations had the severe childhood phenotype, and only a single patient had the mild childhood phenotype. Only 4 of the 18 patients with two missense or single–amino acid deletion mutations had the severe childhood disease phenotype. Interestingly, three of these patients were homozygous for the R429W mutation, suggesting that it is a severe mutation with no residual enzyme activity, like the mutations characterized by Souri et al. (1996, 1998*a*, 1998*b*). The remaining 14 patients with two missense or single–amino acid deletion mutations had the mild childhood phenotype (9 patients) or adult onset of the disease (5 patients).

The clear correlation of genotype with phenotype observed in VLCAD deficiency is in sharp contrast to what is observed in MCAD deficiency, in which we have so far been unable to demonstrate any such correlation (Andresen et al. 1997). This difference may be attributable to the fact that defective long-chain fatty acid oxidation is less well tolerated than defective oxidation of medium-chain fatty acids, because of the greater importance of long-chain fatty acids as fuels, the greater toxicity of long-chain acyl-CoAs (Corr et al. 1989; Fitzsimmons et al. 1997; Paumen et al. 1998), or differences in the efficiency of alternative metabolic pathways for accumulating long-chain and medium-chain fatty acyl-CoAs. This notion is supported by the fact that patients with CPT II deficiency, another defect of long-chain fatty acid oxidation, show a correlation between genotype and phenotype similar to that observed in VLCAD deficiency (Taroni et al. 1993; Verderio et al. 1995; Bonnefont et al. 1996).

An explanation for the observed genotype-phenotype correlation could be as follows: In patients with two null mutations, the complete absence of VLCAD activity will affect many tissues, including the heart and liver, resulting in cardiomyopathy, hepatomegaly, and recurrent episodes of metabolic decompensation (severe phenotype). Patients with missense mutations or single–amino acid deletion mutations may have sufficient residual VLCAD activity, when receiving adequate nourishment, to avoid liver and cardiac symptoms, and may not undertake sufficient sustained exercise, in childhood, to precipitate severe muscle symptoms. During infections or fasting, however, the residual enzyme activity may no longer be sufficient to sustain the increasing demand on hepatic fatty acid oxidation, leading to hypoketotic hypoglycemia and encephalopathy. This notion is supported by the fact that, in all the patients with the mild

childhood phenotype, the disease episodes were triggered by such factors as fasting, vomiting, or fever.

As these patients get older, metabolic decompensation becomes less of a problem, but they also start to undertake more sustained exercise. The residual VLCAD activity may not be sufficient to sustain the demands of exercising muscle, particularly as muscle oxidizes fatty acids in preference to glucose under many circumstances (Kim et al. 1996). This leads to muscle symptoms, such as exercise-induced rhabdomyolysis and myoglobinuria. In six of our patients, who presented with the mild childhood phenotype and are now in their teens or late childhood, the symptoms have changed from episodes of hypoketotic hypoglycemia to muscle soreness, with episodes of myoglobinuria in two patients. All of these six patients have two missense or single–amino acid deletion mutations. The late onset of disease in the adult patients could simply be the result of their avoiding metabolic stress to a sufficient extent, during childhood, to prevent childhood precipitation of disease. Alternatively, it is possible that they have mutations with higher residual enzyme activity than that observed in patients with the mild childhood disease phenotype, resulting in a difference in tolerance of metabolic stress between the two groups of patients.

In addition to the environmental influences mentioned above, other genetic factors may modify the phenotype in VLCAD deficiency. Differences in the cellular handling of mutant proteins, for example, could lead to differences in the residual enzyme activity obtained from identical mutations. Such processes are known to be important in SCAD and MCAD deficiencies (Andresen et al. 1997; Bross et al. 1998; Gregersen et al. 1998). Given that many VLCAD mutations also lead to decreased steady-state protein levels, similar processes may operate in this condition. Finally, because of LCAD's overlapping chain-length specificity, mutations in this enzyme might modify the disease phenotype in VLCAD deficiency. A study by Yamaguchi and coworkers (1993) suggests that a 997A→C mutation in LCAD might have such a role. Our results show, however, that the mutation is polymorphic in the normal population and that it is unlikely to play a significant role in determining the VLCAD phenotype (table 3). This conclusion is consistent with the fact that LCAD is responsible for <10% of the cellular enzyme activity toward palmitoyl-CoA (Aoyama et al. 1995*b*; Vianey-Saban et al. 1998) and seems to have a tissue-specific expression pattern different from that of the other acyl-CoA dehydrogenases in humans (Andresen et al. 1996*a*).

Acknowledgments

Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

EMBL Nucleotide Sequence Database, http://www.ebi.ac.uk/ ebi_docs/embl_db/ebi/topembl.html (for the human VLCAD gene [accession number L46590], mouse VLCAD gene [accession number Z71189], and accession numbers X86556 and AJ012053)

GenBank Nucleotide Sequence Database, http://www.ncbi .nlm.nih.gov/Web/Genbank/index.html (for the human VLCAD gene [accession number L46590], mouse VLCAD gene [accession number Z71189], and accession numbers X86556, AJ012053, and AC003688)

National Center for Biotechnology Information, http://www .ncbi.nlm.nih.gov/BLAST/ (for BLASTn)

Transcription Element Search System, http://agave.humgen .upenn.edu/utess/ (for Transfac 3.4)

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