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ARTICLES

Role of Common Gene Variations in the Molecular Pathogenesis of Short-Chain Acyl-CoA Dehydrogenase Deficiency

MORTEN JUHL CORYDON, JERRY VOCKLEY, PIERO RINALDO, WILLIAM JAMES RHEAD, MARGRETHE KJELDSEN, VIBEKE WINTER, CHARLES RIGGS, DUSICA BABOVIC-VUKSANOVIC, JAN SMEITINK, JAN DE JONG, HARVEY LEVY, ADRIAN CLIVE SEWELL, CHARLES ROE, DIETRICH MATERN, MAJED DASOUKI, AND NIELS GREGERSEN

Research Unit for Molecular Medicine, Aarhus University Hospital and Faculty of Health Sciences, Skejby Sygehus, 8200 Aarhus N, Denmark [M.J.C., M.K., V.W., N.G.]; Department of Medical Genetics, Mayo Clinic and Foundation, Rochester, MN 55905, U.S.A. [J.V., D.B-V.]; Department of Laboratory Medicine & Pathology, Mayo Clinic, Rochester, MN 55905, U.S.A. [P.R.]; Department of Pediatrics, University of Iowa, Iowa City, IA 50011, U.S.A. [W.J.R.]; Human Performance Laboratory, University of Arkansas, Fayetteville, AR 72701, U.S.A. [C.R.]; University Children's Hospital, Department of Metabolic Diseases, 6500 HB Nijmegen, The Netherlands [J.S., J.D.J.]; Division of Genetics, Children's Hospital, Boston, MA 02115, U.S.A. [H.L.]; University Children's Hospital, 60596 Frankfurt am Main, Germany [A.C.S.]; Institute of Metabolic Disease, Dallas, TX 75226, U.S.A. [C.R.]; Division of Medical Genetics, Department of Pediatrics, Duke University Medical Center, Durham, NC 27708, U.S.A. [D.M.]; Children's Mercy Hospital, Kansas City, MO 64108, U.S.A. [M.D.]

ABSTRACT

Short-chain acyl-CoA dehydrogenase (SCAD) deficiency is considered a rare inherited mitochondrial fatty acid oxidation disorder. Less than 10 patients have been reported, diagnosed on the basis of ethylmalonic aciduria and low SCAD activity in cultured fibroblast. However, mild ethylmalonic aciduria, a biochemical marker of functional SCAD deficiency *in vivo*, is a common finding in patients suspected of having metabolic disorders. Based on previous observations, we have proposed that ethylmalonic aciduria in a small proportion of cases is caused by pathogenic SCAD gene mutations, and SCAD deficiency can be demonstrated in fibroblasts. Another - much more frequent - group of patients with mild ethylmalonic aciduria has functional SCAD deficiency due to the presence of susceptibility SCAD gene variations, *i.e.* 625G>A and 511C>T, in whom a variable or moderately reduced SCAD activity in fibroblasts may still be clinically relevant. To substantiate this notion we performed sequence analysis of the SCAD gene in 10 patients with ethylmalonic aciduria and diagnosed with SCAD deficiency in fibroblasts. Surprisingly, only one of the 10 patients carried pathogenic mutations in both alleles, while five were double heterozygotes for a pathogenic mutation in one allele and the 625G>A susceptibility variation in the other. The remaining four patients carried only either the 511C>T or the 625G>A variations in each allele. Our findings document that patients carrying these SCAD gene variations may develop clinically relevant SCAD deficiency, and that patients with even mild ethylmalonic aciduria should be tested for these variations. **(*Pediatr Res* 49: 18–23, 2001)**

Abbreviations:
EMA, ethylmalonic acid
SCAD, short, chain acyl-CoA dehydrogenase

Short-chain acyl-CoA dehydrogenase (SCAD) (EC 1.3.99.2) is the first enzyme of the mitochondrial short-chain β-oxidation spiral catalyzing the dehydrogenation of C_4 and C_6 fatty acids (1). Inherited SCAD deficiency was first reported in 1987 (2). Since then only eight additional patients have been identified on the basis of reduced to absent enzymatic activity *in vitro* (3–10), and the biochemical finding of ethylmalonic aciduria (EMA aciduria), believed to have only limited specificity (11).

Following the report of the SCAD cDNA sequence (12), pathogenic mutations in both alleles of the SCAD gene were identified in one patient with SCAD deficiency (13). Subsequent studies of four unrelated patients with deficiency of SCAD enzyme activity in cultured skin fibroblasts led to the identification of three more pathogenic mutations in the SCAD gene, in addition

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Correspondence and reprint requests: Niels Gregersen, Research Unit for Molecular Medicine, Skejby Sygehus, Brendstrupgaardsvej, 8200 Aarhus N, Denmark.
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to two variations, 625G>A and 511C>T (8, 14, 15). We showed that the 625A and 511T variant alleles, either in homozygous or in double heterozygous form, were over-represented (69% *versus* 14% in the general population) among a clinically heterogeneous group of 133 patients with EMA aciduria. These observations lead to the hypothesis that the SCAD gene variations may represent susceptibility alleles (8, 16).

On the basis of these results, we proposed that patients with EMA aciduria could be divided into two groups: A small group of patients with pathogenic mutations in both alleles of the SCAD gene; and a second, much larger, group of subjects with *in vivo* functional SCAD deficiency who carry any combination of the 625A and 511T variant alleles. These individuals may have normal or moderately reduced enzyme activity *in vitro* under normal conditions, but may become symptomatic under the influence of additional genetic, cellular, and/or environmental factors.

To substantiate the validity of this classification we analyzed the SCAD gene in 10 patients with EMA aciduria and a confirmed SCAD deficiency in cultured skin fibroblast cells. Here we report the results of the mutation analyses in these 10 patients, and discuss the possible implications for the understanding of EMA aciduria and the diagnosis of SCAD deficiency.

METHODS

Subjects

Patients. Clinical and biochemical data of 10 unrelated patients with EMA aciduria and deficient SCAD activity in cultured fibroblasts are summarized in Table 1. Clinical reports of patients 6 and 10 have been published previously (4, 5).

Controls. Lymphoblastoid cells of 50 Danish controls were obtained from the cell bank repository of the Panum Institute

Table 1. *Clinical, biochemical, enzymatic, and molecular data on 10 patients with EMA aciduria and SCAD deficiency*

Patient No.	Gender	Age at onset	Clinical presentation	Urine ethylmalonic acid*	Fibroblast SCAD activity†	Allele 1‡	Allele 2
1	M	3 months	Hypotonia, hypoglycemia Developmental delay	19	Undetectable§	511T	511T
2	F	<1 week	Hypotonia Respiratory distress	Increased‖	Undetectable	**268G>A** 625A	625A
3	M	<1 week	Hypotonia, seizures Developmental delay	Increased‖	Undetectable	**575C>T** 625A	**973C>T** 625A
4	F	<1 week	Dysmorphic features Developmental delay	20	Undetectable	511T	625A
5	M	<1 week	Hypotonia Developmental delay	48,75	Undetectable	**310–312delGAG**	625A
6	F	15 months	Hypotonia	69	12–14%¶, 260%**	625A	625A
7	F	1 month	Hypotonia Developmental delay	22	Undetectable	625A	625A
8	M	3 months	Hypotonia Developmental delay	34,200	10%	**1058C>T**	625A
9	F	<1 week	Hypotonia, seizures Developmental delay	45,68	20%	**1138C>T**	625A
10	F		Low average IQ (identified by newborn screening)	43,74	Undetectable	1147C>T 625A	625A
Controls				<18			

* mmol/mol creatinine.

† Data for patients represent percentage of control short-chain acyl-CoA dehydrogenase (SCAD) activity values after inclusion of anti medium-chain acyl-CoA dehydrogenase − antibodies in crude fibroblast extract. The SCAD activity was determined in all cases at least two times. Activity in extracts from patient 8 and 9 was variable. The reported values in these patients were measured on two occasions; however, in several other preparations, the SCAD activity was undetectable. Mean value ± standard deviation (SD) for controls (five homozygous for the 625G and 511C alleles) was 0.5 ± 0.19 nmol electron-transferring flavoprotein (ETF) reduced/mg protein/min (100 ± 38%).

‡ cDNA numbering starts at the ATG translation initiation. The six new mutations are printed in bold.

§ Undetectable represents <10% of control SCAD activity.

‖ Increased based on qualitative evaluation and reporting of organic acid profile.

¶ Deficient activity in fibroblasts was reported previously (4, 23).

** SCAD activity value from this study.

Mutations in patients 6 and 10 have been reported previously in short (14, 15).

(Copenhagen, Denmark). The appropriate human investigation committees in Denmark approved the use of this control material.

Analytical methods. EMA was determined by standard gas chromatography/mass spectrometry methods. SCAD activity was measured by the anaerobic electron-transferring flavoprotein (ETF) fluorescence-reduction assay (17). Immunoblotting of SCAD in human fibroblasts and *Escherichia coli* extract was performed using a rabbit anti-human SCAD antiserum (18). Genomic DNA was isolated from blood samples and cultured skin fibroblasts by standard methods (19). Fragments covering the SCAD 5′-flanking promoter-region and each of the 10 SCAD gene exons including flanking intron sequences were produced by PCR and sequenced in both directions (20). Total RNA was prepared from frozen patient fibroblasts using a RNAzol kit (WAK-Chemie, Bad Hornburg, Germany), and first-strand cDNA was synthesized from 1 μg of total RNA using a commercial kit (Clontech, Palo Alto, CA). Fragments covering the full-length coding region of the SCAD cDNA were produced using previously described PCR conditions (8). PCR-generated cDNA fragments covering the full-length coding region were cloned into the pGEM-T vector (Promega, Madison, WI) and sequenced.

To express mutant proteins in *E. coli*, mutations were introduced by site-directed mutagenesis into the mature human SCAD cDNA cloned into the pSP64 vector (Promega), then transferred to an expression plasmid (pKK223–3, Pharmacia Biotech, Sweden) (21). Extracts were prepared and analyzed for SCAD enzyme activity and immunoreactive SCAD protein following 4 h induction with isopropylthiogalactoside.

RESULTS

Clinical findings. Contrary to previous reports of highly variable phenotypes associated with SCAD deficiency (3–10), more than half of the patients described in this report showed a phenotype with hypotonia (8/10) and developmental delay (7/10). Hypotonia was noticed shortly after birth in three cases, and within a few months in three additional patients. The oldest patient at the time of diagnosis was 15 month old. Other possible noncoincidental manifestations included hypoglycemia, seizures, dysmorphic features, and hypertonia. There was no correlation between the level of EMA aciduria and the severity of clinical symptoms.

Mutation analysis. Sequence analysis of all exons and flanking intron sequences in the SCAD gene revealed six new potentially pathogenic mutations (printed in bold in Table 1) and one previously reported (8) pathogenic mutation (1147C>T, Table 1) in six of the 10 patients. The 3-bp deletion at position 310–312 of the cDNA sequence of patient 5 is the first deletion described in the SCAD gene. It results in the loss of a glutamine residue at position 80 of the mature SCAD protein. The other mutations were all missense (268G>A, 575C>T, 973C>T, 1058C>T, 1138C>T, and 1147C>T). Four of these were located within a CpG dinucleotide, recognized as a mutation hot-spot (22). The resulting missense mutant SCAD proteins are indicated in Table 2. None of these sequence alterations were present in the SCAD gene from 50 unrelated control individuals, supporting the potential pathogenic nature of the mutations.

The previously identified common SCAD gene variations, 625G>A and 511C>T, which encode the G185S and R147W mutant proteins, respectively, were also found in various combinations with the rare mutations in the 10 patients (Table 1). One patient (patient 3) harbored two rare mutations, five had one mutation (patients 2, 5, 8, 9, and 10), and four had none (patients 1, 4, 6, and 7). The variation 625G>A was found in nine of the patients (Table 1), five of whom were homozygous for this variation, while one was homozygous for the 511C>T variation. Of the five cases homozygous for 625G>A, patients 6 and 7 did not harbor any other mutation in the cDNA sequence. Contrary to previous reports (4, 23), fibroblasts from patient 6 showed normal enzyme activity (Table 1), and had normal amounts of immunodetectable protein as well (results not shown). Patient 4 is likely a double heterozygote for the 511C>T and 625G>A variations, since we have previously shown that these variations are exclusively found in separate alleles (8).

The allelic assignment of the mutations in five of the patients (patients 1, 2, 6, 7, and 10) was obvious because of homozygosity for either the 511C>T or the 625G>A variations. Furthermore, each allele identified in patients 1 and 6 was identified in one of the parents, excluding the possibility of a gene deletion in one allele. To make allelic assignments of the mutations in the remaining five cases, cDNA made from patient fibroblast mRNA was cloned and sequenced. Each mutation was always found in separate clones (between 10 and 19 clones characterized per mutation), confirming the assignment of different mutations in each patient to different alleles.

In conclusion, all 10 patients harbored a rare mutation and/or one of the common variations in each allele of the SCAD gene, thus demonstrating the association between SCAD gene mutations/variations and SCAD deficiency.

Haplotype analysis. In addition to the new mutations and the previously identified missense variations, several additional neutral polymorphic variations were found in this cohort of patients: 321T>C, 990C>T and 1260G>C (24). Haplotypes could be inferred in all patients based on comparison with haplotypes defined in 34 independent control individuals from 17 families (father, mother, child), and the mutations and variations were assigned to a single known haplotype (Table 3) (24).

Sequence analysis of the SCAD 5′-flanking promoter-region in patients and controls revealed a previously unrecognized sequence variation, an C>A substitution at position 171 upstream of the ATG start codon (−171C>A). This variation is present in 38% of control individuals, and thus its functional significance is unclear. Inclusion of this variation in the previously defined haplotypes extended the total number of haplotypes present in the Danish population to 6 (Table 3). The inferred haplotypes of the 10 patients are included in Table 3, and it is obvious that haplotype II, which includes the 625G>A and the −171C>A variations, is highly prevalent among the alleles in the patients as well as in the general population.

Functional analysis of mutations/variations. To evaluate the functional significance of the identified missense muta-

Table 2. *Expression of mutant and wild-type (control) SCAD proteins in* E. coli *at 37°C for 4 h*

Mutation*	Change in mature SCAD protein	Activity (nmol ETF reduced/mg protein/min)†
268G>A	G66S	Undetectable‡
310–312delGAG	E80del	Undetectable
575C>T	A168V	Undetectable
973C>T	R301W	Undetectable
1058C>T	S329L	Undetectable
1138C>T	R356W	Undetectable
511C>T	R147W	4.59
625G>A	G185S	5.75
	Wild type	6.66 ± 0.71

* cDNA numbering starts at the ATG translation initiation and amino acid numbering at the cleavage site between leader peptide and mature protein.

† $n = 3$.

‡ Undetectable represent <0.05 nmol electron-transferring flavoprotein (ETF) reduced/mg protein/min.

tions, the wild-type (control) SCAD mature region, as well as inserts carrying the six rare mutations and the two common variations, were cloned into a prokaryotic expression vector. Following incubation at 37°C for 4 h, crude cellular extracts were prepared and tested for SCAD activity (Table 2). Each of the five new missense mutations and the 3-bp deletion all result in proteins with no detectable enzyme activity, while Western blotting showed the presence of immunoreactive material in all the extracts (data not shown). These results confirm the inactivating nature of these mutations. One of the mutant proteins, R359C (encoded by the 1147C>T mutation), seen in patient 10 has previously been expressed in COS-7 cells where similar results were obtained (8). In contrast, the G185S and R147W variant proteins encoded by the 625A and 511T variant alleles, respectively, showed 86% and 69% activity of the mean wild-type value, respectively, also in agreement with previous expression studies in COS-7 cells at the same temperature (8).

DISCUSSION

We have pursued the molecular characterization of 10 patients with biochemical and enzymatic findings consistent with a diagnosis of SCAD deficiency, an inborn error of metabolism considered to be a rare autosomal recessive disorder (25). In contrast to previous reports, the clinical presentations of the patients in this group were similar although rather nonspecific, with hypotonia and developmental delay as prevalent features. The presence of other very different clinical pictures in these patients (Table 1) and in the previously published cases could indicate a selection bias in the patients described here, and it emphasizes the current difficulties of recognizing patients with this kind of fatty acid oxidation disorder solely on the basis of clinical findings (26).

It is surprising that only one patient (patient 3, Table 1) carried two pathogenic mutations, as indicated by their non-presence in 100 control alleles, and defined by complete loss of enzyme activity of the mutant protein produced in a prokaryotic expression system. The remaining patients were double heterozygous for a pathogenic mutation and the previously identified 625G>A variation, homozygous for one of the variations, 625G>A or 511C>T, or double heterozygous for both of them.

It is noteworthy that four alleles in three of the patients (2, 3, and 10) in addition to a pathogenic mutation carry the 625G>A variation. This could suggest a synergy in the effects, although our present understanding is that the effect of pathogenic mutations eliminates the effect of the variation as indicated by the prokaryotic expression studies. The identification of further similar alleles in patients with SCAD deficiency are needed to evaluate whether they are truly overrepresented, and if so, further cell studies in eucaryotic cells may reveal a possible biologic significance.

The analysis of the haplotypes, defined by the three previously identified neutral SCAD gene variations (321T>C, 990C>T, and 1260G>C), the new variation in the promoter of the SCAD gene (−171C>A), and the 625G>A and 511C>T variations gives a total of 6 haplotypes in the general population (Table 3; the previously defined haplotype I (24) is now divided in IA and IB). Interestingly, the haplotype in the patients with one or two 625G>A variations (designated II) can be identified in 15 of the 68 alleles in the general population, while seven of 68 control alleles are haplotype IB which harbors the 511C>T variation. This indicates that the genetic background - defined by these haplotypes - is not likely to contribute significantly to the apparent pathogenesis related to the 625G>A or 511C>T variations.

In contrast to the severe effect of the six rare mutations on the SCAD enzyme activity, the R147W and G185S variant enzymes showed only a modest reduction in enzymatic activity when produced in *E. coli*. These findings are compatible with previously reported expression studies in COS-7 cells (8), where the enzymatic properties of the two variant proteins were found to be temperature-dependent. In these experiments a shift of temperature of the COS-7 cell expression system

Table 3. *Haplotypes in the SCAD gene in 34 independent control individuals from 17 families (father, mother, child)*

Haplotype	Frequency Denmark*	−171†	321	511	625	990	1260	Identified mutations‡	Frequency 10 patients*
IA	33/68	C	T	C	G	C	G		0/20
IB	7/68	C	T	T	G	C	G		3/20
II	15/68	A	C	C	A	T	C	268G>A; 575C>T; 973C>T; 1147C>T	14/20
III	11/68	A	C	C	G	T	G	310–312delGAG; 1058C>T; 1138C>T	3/20
IV	1/68	C	T	C	G	C	C		0/20
V	1/68	C	T	C	A	C	C		0/20

* Haplotype frequencies in 34 controls (68 alleles) and in the 10 analyzed patients (20 alleles) with EMA aciduria and SCAD deficiency.

† Positions are relative to the start codon of SCAD cDNA.

‡ The pathogenic mutations are assigned to the haplotypes.

from 37°C to 41°C caused a reduction of SCAD activity to 13% and 58% of control activity, respectively, for the R147W and G185S variant proteins, while a reduction of temperature to 26°C led to an increase of activity to 85% and 183% relative to wild-type, respectively. Thus, the activity of the two variant proteins is dependent on cellular conditions, a phenomenon previously documented for other SCAD and medium-chain acyl-CoA dehydrogenase (MCAD) mutant proteins (27–29). In this regard, it is interesting to note that we have found the measurements of SCAD activity in cultured fibroblasts from individuals carrying the variant alleles to be somewhat variable (unpublished results). A striking example of this can be seen in patient 6, who previously was diagnosed with SCAD deficiency based on assay of fibroblast extract (4, 23). The conditions leading to this variability are currently under further investigation. Preliminary results indicate that the amounts of SCAD antigen in fibroblasts from patients carrying the 625A variant allele may vary as much as the enzyme activity, probably reflecting the structural instability of either folding intermediates and/or the final active enzyme.

In total, these findings suggest that the 625G>A and 511C>T variations are unlikely to lead to clinically relevant SCAD deficiency in and of themselves. Rather, we postulate that other genetic, cellular, and environmental factors must be involved in reducing the level of catalytic activity of the variant enzymes below a critical threshold, leading to the onset of clinical symptoms. Depending on the nature of these factors, a deficiency may either be consistently expressed or related to intermittent cellular stress (such as elevated temperature or reduced pH), or variable efficiency of the cellular protein handling system (30).

In retrospect, the possibility that the common variations might predispose to clinical disease was first suggested by the report of a patient who was a double heterozygote for a pathogenic mutation and the 511C>T variation (8). This original interpretation is now convincingly supported by the isolated finding of 511C>T homozygosity in a patient with completely deficient enzymatic activity (patient 1, Table 1). Overall, the two common variations were found in 17/20 (85%) of the alleles in 10 patients with reduced fibroblast SCAD activity. A similar over-representation of these alleles was found in a study of 133 subjects with EMA aciduria (8, 16), indicative of an *in vivo* functional SCAD deficiency, suggesting that SCAD deficiency in fibroblast could probably have been demonstrated in some of these individuals.

The current study shows the need for a substantial revision of our thinking regarding the role played by the 625G>A and 511C>T variations in the etiology of SCAD deficiency. Since these variations are found either in homozygous or double heterozygous form in 14% of the general population (8), the actual incidence of clinically relevant SCAD deficiency could be much higher than currently recognized, possibly becoming one of the most common inborn errors of metabolism known to date. Prospective clinical studies are necessary to establish the true incidence of clinically relevant SCAD deficiency and to define the spectrum of its clinical manifestations from the newborn to the geriatric patient. Until such studies are completed our operative recommendation is to investigate every patient with elevated EMA (>18 μmol/mmol creatinine) for the two variations 625G>A and 511C>T. If either is present in homozygous form or the patient is double heterozygous for them we recommend treatment similar to other fatty acid oxidation deficiencies. If the variations are not present or only in one allele we consider investigation of the whole SCAD gene sequence.

Before we know more about the pathogenesis of the variations and the natural history of children identified with possible SCAD deficiency by the emerging neonatal screening programs for acyl-carnitines, we recommend being very conservative in testing for these variations. As long as we do not know whether other genetic factors contribute to the disease expression and outcome, testing for these variations should be restricted to clinically symptomatic patients of any age with otherwise unexplained manifestations and elevated excretion of ethylmalonic acid.

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