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Clinical relevance of short-chain acyl-CoA dehydrogenase (SCAD) deficiency: Exploring the role of new variants including the first SCAD-disease-causing allele carrying a synonymous mutation

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ABSTRACT

Short-chain acyl-coA dehydrogenase deficiency (SCADD) is an autosomal recessive inborn error of mitochondrial fatty acid oxidation caused by *ACADS* gene alterations. SCADD is a heterogeneous condition, sometimes considered to be solely a biochemical condition given that it has been associated with variable clinical phenotypes ranging from no symptoms or signs to metabolic decompensation occurring early in life.

A reason for this variability is due to SCAD alterations, such as the common p.Gly209Ser, that confer a disease susceptibility state but require a complex multifactorial/polygenic condition to manifest clinically.

Our study focuses on 12 SCADD patients carrying 11 new *ACADS* variants, with the purpose of defining genotype–phenotype correlations based on clinical data, metabolite evaluation, molecular analyses, and *in silico* functional analyses.

Interestingly, we identified a synonymous variant, c.765G>T (p.Gly255Gly) that influences *ACADS* mRNA splicing accuracy. mRNA characterisation demonstrated that this variant leads to an aberrant splicing product, harbouring a premature stop codon.

Molecular analysis and *in silico* tools are able to characterise *ACADS* variants, identifying the severe mutations and consequently indicating which patients could benefit from a long term follow- up. We also emphasise that synonymous mutations can be relevant features and potentially associated with SCADD.

1. Introduction

Short-chain acyl-CoA dehydrogenase (SCAD) deficiency (SCADD, MIM 201470) is an autosomal recessive inborn error of mitochondrial fatty acid oxidation with highly variable clinical, biochemical and genetic characteristics [1]. SCADD is one of the most common inborn errors of metabolism since it is estimated to affect approximately 1 in 35,000 to 50,000 newborns [1–2].

The characteristic manifestations of SCAD (MIM 606885) deficiency include hypoglycaemia, weakness hypotonia and lethargy [3]

Biochemically, SCADD is associated with the accumulation of butyrylcarnitine (C4-C), butyrylglycine, ethylmalonic acid (EMA), and methylsuccinic acid in blood, urine and cells [4]. Blood C4-C and urinary EMA are generally elevated and thus are currently used as biochemical markers of the disease [1].

SCAD is a flavoprotein consisting of four subunits, each of them containing one molecule of the flavin adenine dinucleotide cofactor (FAD). FAD binding is important for the catalytic activity of flavoproteins, as well as their folding, assembly, and/or stability [5–6].

Abbreviations: SCAD, Short-chain acyl-CoA dehydrogenase; SCADD, Short-chain acyl-CoA dehydrogenase deficiency; C4-C, butyrylcarnitine; EMA, ethylmalonic acid; NBS, Newborn screening; LC–MS/MS, Tandem mass spectrometry; *ACADS*, Acyl CoA-deydrogenase, short chain.

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Clinically, SCADD is usually diagnosed as a result of investigations for developmental delay, epilepsy, behavioural disorders, hypoglycaemia, and hypotonia.

Newborn screening (NBS) using tandem mass spectrometry (LC–MS/MS) has led to the identification of an increasing number of newborns with altered biochemical markers suggesting SCADD. However, in contrast to infants in whom clinical manifestations are a cause of referral, infants brought to medical attention by NBS programmes could remain largely asymptomatic [1,7].

The diagnosis of SCADD is usually confirmed by molecular analysis. The *ACADS* gene (MIM #606885), encoding SCAD, spans approximately 14.2 kb and consists of ten exons. To date, more than 60 inactivating mutations have been reported in the *ACADS* gene, two of which, the c.511C>T (p.Arg171Trp) and c.625G>A (p.Gly209Ser), are polymorphic and generally referred to as gene variants. These two variants have not been directly associated with SCADD although they were reported to confer disease susceptibility when co-occurring with as yet undetermined environmental or genetic factors [8–9]. Assuming that these *ACADS* variants result in protein misfolding [10], riboflavin (the precursor of FAD) therapy might be efficacious. However, riboflavin responsiveness in individuals with the c.625G>A (p.Gly209Ser) variant at a homozygous state, was detected only in combination with an initial low FAD status observed in patients' blood samples [11].

The purpose of this study is to increase the knowledge on the clinical relevance of SCADD. We focus on 12 cases of SCADD exhibiting a new genotype and report their clinical, biochemical and molecular data. We detected 11 new mutations in the *ACADS* gene including the first synonymous (silent) mutation in a SCADD disease causing allele. We evaluated the pathogenic role of the new missense variants identified using *in silico* predictions and performed RT-PCR analysis to study the effects of the synonymous variant.

2. Materials and methods

2.1. Clinical and biochemical analysis

This study was carried out on 12 patients from 11 unrelated families.

Clinical features, the EMA and C4-C values and *ACADS* gene molecular analyses are reported in Table 1.

According to ethical guidelines, all cell and nucleic acid samples were obtained for analysis and storage after patients' (and/or parental) written informed consent, using a form approved by the local Ethics Committee.

Quantitative assay of acylcarnitine was performed by tandem mass spectrometry (LC/MS/MS) using an ABiSciex API 4000 triple-quadruple mass spectrometer equipped with a TurboIonSpray source (ABiSciex, Toronto, Canada) as previously reported [12].

2.2. Analysis of genomic DNA

Genomic DNA was isolated from peripheral blood lymphocytes or cultured fibroblasts, or both.

Amplification of genomic fragments was performed on 200 ng of genomic DNA; PCR conditions for all the *ACADS* exons included denaturation at 94 °C for 4 min, 30 cycles at 94 °C for 30 s, 63 °C for 30 s, 72 °C for 2 min, and a final extension cycle at 72 °C for 10 min.

PCR products were visualised on a 2% agarose gel and purified using Nucleospin Extract II extraction kit (Macherey-Nagel, Düren, Germany). About 100 ng of purified DNA was analysed for mutation detection by nucleotide sequencing on an ABI PRISM 3130 genetic analyser using BigDye terminator chemicals (Life Technologies Italia, Monza, Italy).

2.3. Cell culture

T lymphocytes were cultured in RPMI medium added by foetal bovine serum (heat inactivated for 30 min at 56 °C), interleukin 2 (800 μ/ml), phytohemagglutinin (2.5 μg/ml) and antibiotics.

2.4. mRNA analyses

Total RNA was isolated from T lymphocytes cells by using the RNeasy mini kit (Qiagen, Hilden, Germany). RNA integrity and concentration were checked by 1% agarose gel and Nanodrop® ND-1000 Spectrophotometer (Nanodrop technologies, Wilmington, USA).

Total RNA (200 ng) was reverse transcribed with random hexamers by using TaqMan Reverse Transcriptase kit (Applied Biosystems by Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions.

Table 1
Genotype, biochemical values and clinical features of the here reported 12 SCADD patients.

Patient	*ACADS* gene Allele 1	*ACADS* gene Allele 2	Age at diagnosis	Ethnic origin	C4-C μmol/L (DBS) (n.v < 1)	EMA mmol/mol creatinine (n.v. < 7)	Clinical symptoms
1	**c. 842G>C p.Gly281Ala c.625G>A p.Gly209Ser**	c.625G>A p.Gly209Ser	5 y	Italian	–	48	Hypotonia, developmental delay, epilepsy, dysmorphic features, microcephaly and maculopathy
2	**c.869C>G p.Ala290Gly** c.625G>A p.Gly209Ser	c.625G>A p.Gly209Ser	26 d	Italian	n.a	42	None
3	**c.981_983delGAC**	c.322G>A p.Gly108Ser	15 d	Philippine	1.05	154	Mild hypotonia, mild hypertransaminasaemia
4	**c.1054G>A p.Ala352Thr** c.625G>A p.Gly209Ser	**c.1054G>A p.Ala352Thr** c.625G>A p.Gly209Ser	9 d	Moroccan	4	182	none
5	**c.1130C>T p.Pro377Leu**	c.625G>A p.Gly209Ser	17 y	Italian	–	14	Pervasive developmental disorder, stereotypies, dysmorphic features
6	**c.700C>T p.Arg234Trp**	c.625G>A p.Gly209Ser	9 d	Moroccan	1.59	33	None
7	**c.1157G>A p.Arg386His** c.625G>A p.Gly209Ser	c.625G > A p.Gly209Ser	8 d	Romanian	1	28	None
8	**c.814C>T p.Arg272Cys**	**c.814C>T p.Arg272Cys**	16 d	Moroccan	1.87	187	Mild hypotonia
9	**c.814C>T p.Arg272Cys**	**c.814C>T p.Arg272Cys**	8 d	Moroccan	1.80	96	Mild hypotonia
10	**c.66G>A p.W22***	c.625G>A p.Gly209Ser	8 d	Pakistani	1.13	36	None
11	**c.700C>T p.Arg234Trp**	**c.700C>T p.Arg234Trp**	14 d	Italian	2.25	220	Mild hypotonia
12	**c.531 G>A p.Trp171***	**c.765G>T p.Gly255Gly**	9 d	Italian	1.98	129	None

– not performed: they were born before NBS programmes were established in our country; n.a. not available; y = years; d = days; n.v = normal values. The new variants are bolded. Patients 8 and 9 are brothers.