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SUCLA2 mutations are associated with mild methylmalonic aciduria, Leigh-like encephalomyopathy, dystonia and deafness

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One pedigree with four patients has been recently described with mitochondrial DNA depletion and mutation in *SUCLA2* gene leading to succinyl-CoA synthase deficiency. Patients had a Leigh-like encephalomyopathy and deafness but besides the presence of lactic acidosis, the profile of urine organic acid was not reported.
We have studied 14 patients with mild 'unlabelled' methylmalonic aciduria (MMA) from 11 families. Eight of the families are from the Faroe Islands, having a common ancestor, and three are from southern Italy. Since the reaction catalysed by succinyl-CoA synthase in the tricarboxylic acid (TCA) cycle represents a distal step of the methylmalonic acid pathway, we investigated the *SUCLA2* gene as a candidate gene in our patients. Genetic analysis of the gene in the 14 patients confirmed the defect in all patients and led to the identification of three novel mutations (p.Gly118Arg; p.Arg284Cys; c.534 + 1G → A). The defect could be convincingly shown at the protein level and our data also confirm the previously described mitochondrial DNA depletion. Defects in SUCLA2 can be found at the metabolite level and are defined by mildly elevated methylmalonic acid and C4-dicarboxylic carnitine concentrations in body fluids in association with variable lactic acidosis. Clinically the diagnosis should be considered in patients with early/neonatal onset encephalomyopathy, dystonia, deafness and Leigh-like MRI abnormalities mainly affecting the putamen and the caudate nuclei. The frequency of the mutated allele in the Faroese population amounted to 2%, corresponding with an estimated homozygote frequency of 1 : 2500. Our data extend knowledge on the genetic defects causing MMA. Our patients present with an early infantile Leigh-like encephalomyopathy with deafness, and later on a progressive dystonia. Mild MMA, lactic acidosis and specific abnormalities in the carnitine ester profile are the biochemical hallmarks of the disease. In view of the frequency of the mutated allele on the Faroe Islands, measures become feasible to prevent the occurrence of the disease on the islands. We confirm and extend the findings on this inborn error of metabolism in the TCA cycle that must be carefully investigated by accurate metabolite analyses.

Keywords: methylmalonic aciduria; succinylcarnitine; mtDNA depletion; succinyl-CoA synthetase; TCA cycle defect

Abbreviations: MIM = Mendelian Inheritance in Man database; MMA = methylmalonic aciduria; mtDNA = mitochondrial DNA; SCS-A = ADP-forming succinyl-CoA synthetase; SCS-G = GDP-forming succinyl-CoA synthetase; *SUCLA2* = gene coding for the β-subunit of ADP-forming succinyl-CoA synthetase; TCA = tricarboxylic acid; GC–MS = gas chromatography/mass spectrometry; MSMS = tandem mass spectrometry

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Introduction

Methylmalonic aciduria (MMA) is one of the most frequent forms of branched-chain organic acidurias, a class of diseases caused by enzymatic defects involved in the catabolism of branched-chain amino acids valine and isoleucine and of other propiogenic substrates such as methionine, threonine, odd-chain fatty acids and cholesterol. MMAs encompass a group of genetically heterogeneous autosomal recessive disorders of methylmalonate and cobalamin metabolism caused by a defect in the conversion of methylmalonyl-CoA to succinyl-CoA (Deodato *et al.*, 2006). Patients with isolated methylmalonic acidaemia may have a defect of the apoenzyme methylmalonyl-CoA mutase (MIM 251000). Isolated MMA also occurs in patients with defects in the synthesis of the co-factor adenosylcobalamin, cblA (MIM 251100) and cblB (MIM 251110). The diagnosis of MMAs is based on the presence of characteristic compounds in body fluids as detected by urine organic acid analysis and blood acylcarnitine profiling. Patients show massively increased urinary excretion of methylmalonic acid accompanied by increased excretion of methylcitric acid, 3-hydroxypropionic acid and other derivatives of propionyl-CoA. Propionyl and methylmalonyl carnitine are the predominant abnormal carnitine esters in blood of these patients.

In most cases the onset of the disease is in the neonatal period with recurrent vomiting, dehydration, respiratory distress, hypotonia, progressive lethargy, seizures and coma, leading to death if not promptly treated. In the later-onset form, the clinical picture is more variable, ranging from acute life-threatening encephalopathy to intermittent or chronic symptoms of various degrees. The classical form caused by apoenzyme mutations is indistinguishable from the two variants due to defects in adenosylcobalamin formation; however, the long-term prognosis is less severe in co-enzyme defects because of vitamin B12 responsiveness. The neurological picture in patients with MMA is frequently characterized by an acute and progressive dystonic syndrome resulting from basal ganglia lesions. These are localized bilaterally in the globus pallidus and usually occur following acute episodes of metabolic decompensation.

Increased levels of methylmalonic acid can also be observed in MMA with homocystinuria, caused by other defects of cobalamin metabolism (cblC, MIM 277400, and cblD, MIM 277410) which impair the conversion of vitamin B12 into its two metabolically active forms, methylcobalamin and adenosylcobalamin. However, these forms present with a different clinical and brain MRI phenotype (Longo *et al.*, 2005). Very recently, a new genetic defect caused by mutation in the methylmalonyl-CoA epimerase gene has been reported in patients with mild MMA (MIM 251120) (Bikker *et al.*, 2006; Dobson *et al.*, 2006).

Besides these clinically, biochemically and genetically well-defined conditions, there are a few reports describing 'unlabelled' MMA cases, presenting with progressive encephalopathy, bilateral symmetric lesions of basal ganglia and mild urinary excretion of methylmalonic acid (Stromme *et al.*, 1995; Mayatepek *et al.*, 1996; Deodato *et al.*, 2005). In these cases, all known forms of MMA were excluded, suggesting a hitherto unrecognized metabolic disorder(s). Furthermore, mild MMA has been reported in two unrelated infants with cortical atrophy of the frontal lobes, metabolic acidosis and mitochondrial DNA depletion (Yano *et al.*, 2003).

Recently, mutations in the gene encoding the β-subunit of the ADP-forming succinyl-CoA synthetase (SCS-A; MIM 603921; EC 6.2.1.5) (*SUCLA2*) have been reported in an inbred family in whom two patients had mitochondrial DNA depletion in association with a Leigh-like encephalomyopathy. Besides the presence of lactic acidosis, the profile of urine organic acid in those children was not reported (Elpeleg *et al.*, 2005). Succinyl-CoA synthetase catalyses the reversible synthesis of succinate and ATP from succinyl-CoA and ADP in the tricarboxylic acid (TCA) cycle (Fig. 1).

This reaction that represents a distal step of the methylmalonic acid pathway, led us to investigate the *SUCLA2* gene in a series of patients with 'unlabelled' MMA presenting clinical similarities with the patients described by Elpeleg *et al.* (2005).

Material and methods

Subjects

We selected three patients from three southern Italian families (mother of patient 2 is from Romania), and 11 patients from an

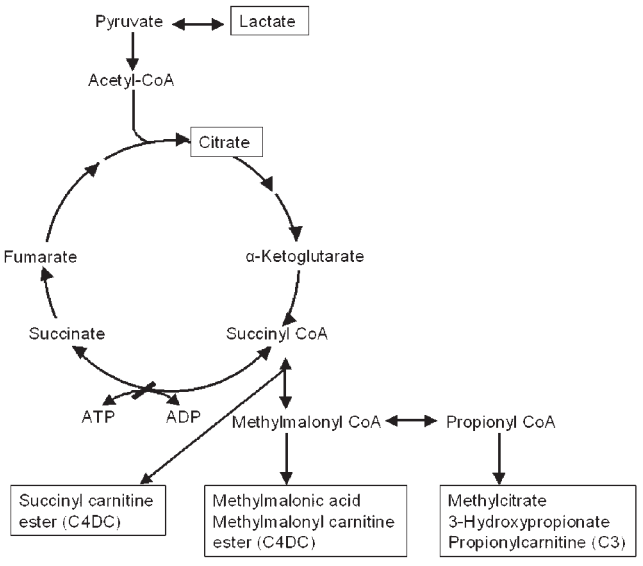

Fig. 1 Relevant metabolic pathways illustrating the metabolic effects of ADP-forming succinyl-CoA synthetase deficiency.

inbred kinship in the Faroe Islands where common ancestors could be traced back to the period 1630–50. For privacy reasons a simplified family tree is shown (Fig. 2). The 11 Faroese patients are from eight families with three families having two affected children. Genome scanning was performed in this pedigree. In addition, we also reviewed the literature of other 'unlabelled' MMA and analysed six patients previously described [Stromme *et al.*, 1995; Yano *et al.*, 2003 (patient 1), Deodato *et al.*, 2005].

Patient 1

This is the third child of healthy parents coming from a small village in southern Italy. The family history is unremarkable. The patient was born at term by normal delivery after an uneventful pregnancy with a birth weight of 3.5 kg. Since the first months of life retardation of gross motor development and severe axial hypotonia were observed. Feeding problems and gastroesophageal reflux were noted from the first weeks of life. At the age of 8 months he was hospitalized for evaluation. Length, weight and head circumference ranged between 50th and 75th percentile. There was axial hypotonia, no head control, poor spontaneous movements, and deep tendon reflexes were difficult to elicit. The child was alert and able to smile, and grossly his mental capacity was better than his motor function. Electroencephalography (EEG), nerve conduction velocity (NCV), fundoscopy and visual evoked potentials (VEP) were normal, while brainstem auditory evoked potentials (BAEP) revealed neurosensorial deafness. Routine laboratory investigations showed metabolic acidosis and increased blood lactate (4–6 mmol/l, normal <2). Ammonia, amino acids, total homocysteine, folates and vitamin B12 were normal in blood. At short-term follow-up gross motor dysfunction persisted, while intermittent dystonic movements of the arms started to appear at the age of 1 year.

Patient 2

This child is the second born from non-consanguineous healthy parents; the father is from southern Italy and the mother from Romania. His eldest sister is healthy. Pregnancy and delivery (Apgar score 8/9) were uneventful. At birth, length, weight and head circumference were normal. Delayed motor milestones and failure to thrive were noticed from the age of 4 months. Thereafter, the child has never been able to sit alone, and when evaluated at age 14 months he was alert, was able to control his head but did not sit on his own because of axial hypotonia and weakness, and had no rolling when laid supine. He was able to catch an object and bring it to his mouth without dystonic posturing. Laboratory investigations showed increased blood lactate (7.5 mmol/l, normal <2). Ammonia, amino acids, total homocysteine, folates and vitamin B12 were normal in blood.

Patient 3

This patient is distantly related to patient 2: his mother is a second-degree cousin of the father of patient 2, the proband is the second born and his elder sister is apparently well. Delayed motor milestones and failure to thrive were noticed from the fourth month of life. At the age of 3 years the child was alert and able to smile, he presented poor growth, axial hypotonia, dystonic movements and weak tendon reflexes. BAEP revealed neurosensorial deafness. Laboratory investigations showed increased blood lactate (3.0 mmol/l, normal <2). Ammonia, amino acids, total homocysteine, folates and vitamin B12 were normal in blood.

Patients 4–14

The 11 probands were born to healthy parents from eight related families after uneventful pregnancies; two were born by Caesarian section. No dysmorphic features were present at birth. Feeding problems were noted mostly from the neonatal period, leading to failure to thrive. The children also suffered from recurrent upper respiratory infections becoming less apparent after school-age. The patients had a severe muscle hypotonia with progressive areflexia. There was a profound motor developmental delay, none of the patients learned to sit or stand without support. No speech development was observed but the children learned to understand

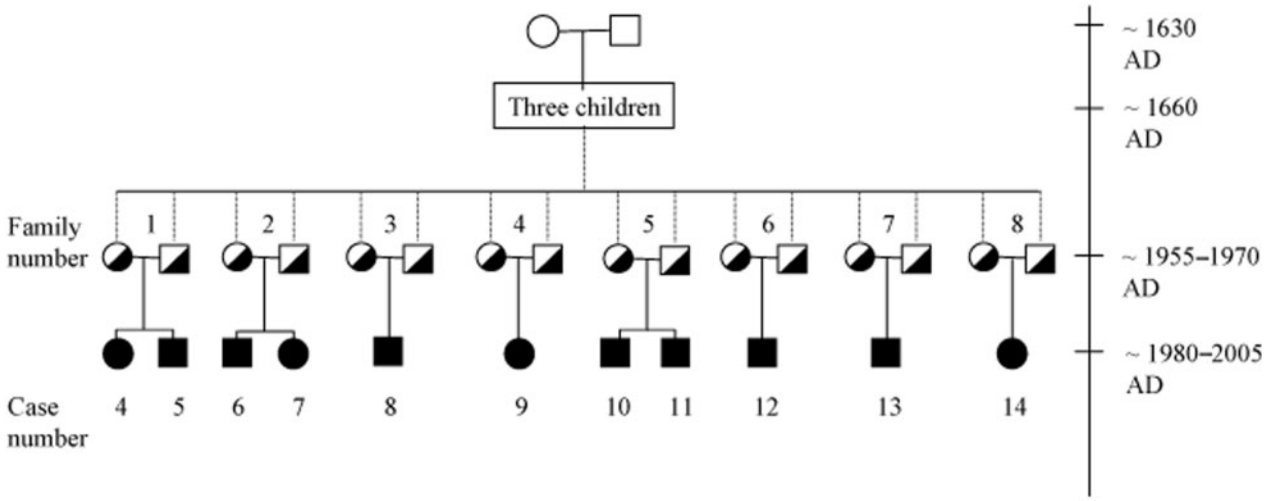

Fig. 2 Faroese family tree (patient numbers 4–14) The 11 patients have a common ancestor in the period 1630–1750. A detailed family tree covering the period 1650–1955 is available to the authors but not shown for privacy reasons. For the same reason only affected cases are shown in the figure.

sign language. Due to the progressive dystonia the slow motor development arrested with a progressive neurological deterioration from the age of 12–14 months. The patients developed a hyperkinetic–dystonic movement disorder and external ophthalmoplegia and/or ptosis. Divergent strabismus occurred later in the course of the disease after the age of 1 year. All patients were diagnosed with profound neurosensorial deafness as detected by BAEP studies. Three received a cochlear implant with improvement in their communication. After the implant they were able to understand spoken language. Two had a progressive axonal and demyelinating polyneuropathy, especially of the lower limbs, confirmed by nerve conduction studies. One had epilepsy, one had a very mild restrictive cardiomyopathy and one a renal tubular dysfunction with features of Fanconi syndrome. Six patients died in the paediatric age and one in young adulthood due to intercurrent infections. Eight of the eleven patients had a CT-scan or MRI or both.

Biochemical and molecular analyses

Urine organic acids and plasma acylcarnitines were analysed according to standard methods. Briefly, organic acids were analysed by gas chromatography/mass spectrometry (GC–MS) after extraction with ethyl acetate and derivatization with *N*,*N*-bis(trimethylsilyl)trifluoracetamide containing 1% trimethylchlorosilane. Acylcarnitine esters were measured by tandem mass spectrometry (MSMS) after butylation, essentially as previously described (Vreken *et al*., 1999; Rizzo *et al*., 2003). Methylmalonic acid in plasma was measured by liquid chromatography MSMS. Briefly, 100 µl of body fluid was mixed with deuterated stable isotope-labelled methylmalonic acid (to counter effect any matrix and ion suppression effects), and deproteinized with an ultracentrifugation tube (molecular mass cutoff: 30 kDa). An acidified aliquot was injected onto a C18 column for chromatographic separation of methylmalonic acid and succinic acid. The MSMS was operated in negative multiple reaction monitoring mode measuring the carbonyl loss of both compounds. Quantification was performed by peak area comparison using calibration curves. NMR spectroscopy of urine and plasma was performed essentially as described before (Wevers *et al*., 1994).

Spectrophotometric determination of respiratory chain enzyme complex activities in muscle homogenate (measured in three patients), determination of the mtDNA/18S rRNA ratio in tissues by Southern blot analysis, SDS electrophoresis/western blotting, total DNA purification and PCR amplification was performed using previously reported methodologies (DiMauro *et al*., 1987; Carrozzo *et al*., 2003; Janssen *et al*., 2006).

The activity of SCA-A was measured as described before (Cha and Parks, 1964; Elpeleg *et al*., 2005) in a mitochondria-enriched 600 g supernatant of a muscle homogenate prepared in 0.25 M sucrose, 2 mM EDTA, 5.104 U/l heparin, 10 mM Tris-Cl pH 7.4. The activity was measured in the direction of succinyl-CoA formation either in the presence of ATP (for SCS-A) or GTP (for SCS-G). The ratio ΔAbs/min measured with either ATP or GTP present was used to determine the relative activity of the ADP-forming succinyl-CoA.

Immunolabelling of the *SUCLA2* gene product used a polyclonal antibody (1 : 1000) kindly provided by Prof. David O. Lambeth. The anti-complex II-70 kDa antibody (0.1 µg/ml), used as internal control, was purchased from Mitosciences (Eugene-OR, USA). Reactive bands were detected using the Immobilon Western Chemiluminescent HRP Substrate detection kit (Millipore Corporation, Billerica, MA, USA). Fluorescence was quantified using the Quantity One Software (BioRad, Hercules, CA, USA).

The coding exons of the *SUCLA2* gene and the flanking intronic sequences (Genebank accession no. NM_003850) were amplified using intronic primers (sequences and PCR conditions are available by e-mail: carrozzo@opbg.net) and the BigDye 3.1 chemistry (Applied Biosystems, Foster City, CA, USA). Numbering of bases is according to the approved nomenclature, with the A of the first methionine as base + 1 (Antonarakis, 1998). Mutations were confirmed by resequencing a newly amplified PCR product. Segregation of the mutation within the families as well as the analysis of at least 200 control alleles was performed with *ad hoc* designed PCR–restriction fragment length polymorphism (PCR/RFLP) strategies. Oligonucleotide primers forward (5′→3′) AAGATCAAATTTGACTCTAAATCAGCCAAT (mismatched bases are underlined) and backward (5′→3′) ATTAAACTTAGTGAA TCCAAT were used to demonstrate the presence of p.Arg284Cys mutation in patients 1 and 3, its segregation within the family of patient 1 as well as its absence in controls. The 189-bp PCR amplified fragment is normally cleaved by the *Tsp*509 I endonuclease into fragments sized 166, 17 and 6 bp whereas the presence of the p.Arg284Cys introduces an additional cleavage site producing fragments sized 145, 21, 17, and 6 bp. The segregation of the p.Gly118Arg mutation in patient 2, its segregation within his family as well as the absence in controls used oligonucleotide primers forward (5′→3′) GCTGCTCCATGCTTCATATTT and backward (5′→3′) TCCCGTATGTATCATGTCTAC. The heterozygous p.Gly118Arg introduces a single cleavage site for the endonuclease *Ear* I and produces fragment sized 398 and 117 bp. The segregation of the c.534 + 1G → A mutation within the Faroese families was assessed using oligonucleotide primers forward (5′→3′) AACAATGGAAAGGTCATTTTAA (mismatched base is underlined) and backward (5′→3′) ATGCTCATGACATA CAAACAG. The 154-bp PCR amplified fragment is normally cleaved by the endonuclease *Dra* I into fragments sized 115 and 39 bp. The splice site mutation introduces an additional *Dra* I cleavage site resulting in fragments of 95, 39 and 20 bp.

To assess the functional effect of the c.534 + 1G → A mutation, polyA+ RNA was purified from cultured skin fibroblasts and reverse-transcribed to cDNA using the First Strand cDNA Synthesis kit (Roche Diagnostics SpA, Milano, Italy). Oligonucleotide primers 5′-CCGAGGGGACGGGGTCCGA-3′ and 5′-ATTTAACACCTTCAGCCATCCA-3′ were used to amplify the entire *SUCLA2* cDNA. To sequence the two major cDNA fragments of 1.1 and 1 kb, detected in patient 12, the reverse primer 5′-GAGCTTGTTCCTTTTTGATGCC-3′ was also used.

DNA from five Faroese patients was included in a whole-genome scan. Two of the patients included were sibs. The Affymetrix GeneChip Mapping 10K Set (Affymetrix UK Ltd., High Wycombe, UK) containing 10 204 single neutral polymorphisms (SNPs) with a mean intermarker distance of 258 kb and an average SNP heterozygosity of 0.38 was used for homozygosity mapping. Regions in which all 5 individuals were homozygous for the same allele and which were either 2 cM or larger in size and/or included ⩾10 homozygous SNPs were selected for further study.

To investigate the allele frequency of the mutated gene in the Faroe Islands, DNA was extracted from neonatal blood spots (Guthrie cards) as described (Santer *et al*., 2001). Restriction enzyme analysis using *Mae*III was used (primer sequences and PCR conditions are available from Dr Santer upon request).

Results

Clinical findings, brain imaging and spectroscopy

Table 1 summarizes the relevant clinical and neuroradiological data obtained in the patients.

Onset and progress of the disease were similar in all patients. Clinically the disease in the patients can be characterized as an early neonatal onset encephalomyopathy with dystonia and deafness. Figure 3 illustrates the MRI of the brain of patients 1 (A–E) and 2 (G–I), at the age of 13 months and 12 months, respectively, showing symmetric T_2-hyperintense lesions of putamen and caudate nuclei. In patient 1, the first MRI at 8 months of age showed enlarged subarachnoidal spaces (data not shown) while the second examination at 13 months clearly illustrated deterioration with brain atrophy and involvement of putamen and caudate nuclei (Fig. 3A–E). Proton MRS of the brain in patient 1 showed the presence of lactic acid in the basal ganglia (Fig. 3F). Brain MRI of patient 3 at the age of 3 years showed symmetric T_2-hyperintense lesions of putamen and caudate nuclei. Early MRIs of 3 Faroese patients at the age of 4, 6 and 14 months (case 9, 11 and 12, respectively) also showed slightly enlarged subarachnoidal spaces and ventricular system. The supratentorial white matter and brainstem were normal. At 14 months (case 12) there were clear symmetric T_2-hyperintensive lesions in the putamen, globus pallidus and the caudate nucleus, with the putamen being the most affected. The same abnormalities were also observed, but less pronounced at 6 months of age (case 11) but were absent in case 9 at 4 months of age. Basal ganglia involvement was also observed in CT scans of patients 5 and 6. The MRI findings were confirmed at obduction of patient 8 who died at the age of 15.5 years. Post-mortem findings in this child were generalized brain atrophy with severe dilatation of the ventricles and decreased amounts of white matter. There was extreme atrophy of the basal ganglia with a nucleus caudatus of 1–2 mm only. There were multiple cysts in the nucleus lentiformis along the capsula externa. In the mesencephalon small pedunculi were observed. There were no further abnormalities in the brainstem with a normal nucleus olivares. Also the nucleus dentatus was unremarkable.

Biochemical investigations at the metabolite level

Metabolite findings were remarkably similar in the patients (Table 2). An obvious increase of methylmalonic acid was documented in plasma and to a lesser extent in the urine of the patients (Table 2). This finding was also confirmed with proton NMR spectroscopy. Plasma homocysteine was always normal (data not shown). Lactic acidosis was present in almost all patients although variable over the day and according to the clinical conditions. Lactate values as high as 11.4 mmol/l have been documented. High plasma alanine was a frequent finding. Lactate elevations (>3000 μmol/l) were also documented in CSF of several patients. GC–MS analysis of urinary organic acids showed increased levels of some citric acid intermediates in the patients. Methylcitrate and 3-hydroxypropionate were also detectable in some cases. These findings prompted NMR spectroscopy evaluations of urine samples from five patients (Table 2). Surprisingly succinic acid, the product of the defective enzyme, was slightly increased in urine of the patients (range: 120–210; reference 5–81 μmol/mmol creatinine), but not in plasma. A bacterial origin of the succinate is highly unlikely as this was a constant observation in all investigated patients. Also urinary citric acid was obviously increased (range: 690–1500 with only 1 sample <1250; reference 120–582 μmol/mmol creatinine). In some patients α-ketoglutaric acid and fumaric acid were found intermittently increased.

Carnitine ester profiling in plasma showed increased C3-carnitine and C4-dicarboxylic carnitine (Table 2). The excretion of the C4-dicarboxylic carnitine in urine was even more pronounced (around 20 times increased). The C4-dicarboxylic carnitine ester in these patients is likely to be a mixture of the succinyl- and the methylmalonyl carnitine ester.

Biochemical investigations at the protein level

Muscle respiratory chain enzyme activities in patient 1 showed a combined deficiency of the complexes I (50%), III (44%) and IV (36%). Patient 2 had a reduction of 46% of complex IV and low–normal activity of complex I. In the Faroese patient 13, a combined deficiency of complex I and IV (69 and 43%, respectively) was found in a fresh muscle biopsy sample. All values are normalized for citrate synthase activity and expressed as percentage of the lowest control values. In summary a picture emerges for these three patients of a combined deficiency of variable severity for the complexes I and IV of the respiratory chain.

The SCS-A activity in muscle mitochondria from patient 12 was reduced compared with controls (0.59 U/U SCS-G; control range: 0.86–1.33; $n = 5$) which is 55% of the mean control activity.

Western blotting of the *SUCLA2* gene product in muscle homogenate of patients 1, 2 and 12 showed a reduction to 41, 34 and 81%, respectively, compared with control when normalized on immunodetection of SDH-70 (Fig. 4).

Molecular genetic investigations

Mutation analysis of the *SUCLA2* gene on genomic DNA showed a homozygous c.850C → T mutation (p.Arg284Cys) in exon 7 in patient 1 and 3. Parents and a healthy sib of patient 1 were heterozygous for this mutation. An older brother did not have a mutated allele. Patient 2 was a compound heterozygote. In one allele he carries the c.850C → T mutation, that was present in the father. In the second allele a mutation c.352G → A (p.Gly118Arg) in exon 3

Table 1 Clinical signs and symptoms of the patients

Patients	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Sex	m	m	m	f	m	m	f	m	f	m	m	m	m	f
Ethnicity	Italian	Italian/ Romanian	Italian	Faroese fam 1	Faroese fam 1	Faroese fam 2	Faroese fam 2	Faroese fam 3	Faroese fam 4	Faroese fam 5	Faroese fam 5	Faroese fam 6	Faroese fam 7	Faroese fam 8
Age at onset	3 months	4 months	4 months	Birth	Birth	Birth	Birth	Birth	3 months	Birth	Birth	3 months	Birth	4 months
Present age/ died (years)	2	1.2	6	† 0.8	† 21.5	† 18.5	† 9.5	† 15.5	16.5	† 10.5	6.5	11	† 4.5	3
Delayed motor development	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Muscle hypotonia	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Dystonic/athetoid movements	±	±	+	−	+	+	+	+	+	+	+	+	+	+
Progressive spasticity	−	−	+	−	+	+	+	+	+	+	+	+	+	+
Psychomotor retardation	+	+	+	NA	+	+	+	+	+	+	+	+	+	+
Ophthalmoplegia/ strabismus/ptosis	−	−	−	−	+	+	+	+	+	+	+	+	+	+
Sensorineural hearing loss	+	NA	+	+	+	+	+	+	+	+	+	+	+	+
Peripheral neuropathy	−	NA	−	NA	+	+	NA	+	NA	+	NA	+	NA	NA
Failure to thrive	−	+	+	−	+	+	+	+	+	+	+	+	+	+
Feeding problems and gastrointestinal symptoms	+	+	+	−	+	+	+	+	+	+	+	+	+	+
Lactic acidaemia	+	+	+	NA	−	NA	+	+	+	+	+	+	−	+
Cerebral atrophy (MRI/CT/post-mortem)	+	+	−at 3 years	NA	+	+	NA	+	−at 4 months	+/−	+∗	+	NA	+∗
Basal ganglia involvement (MRI/CT/post-mortem)	+	+	+	NA	+	+	NA	+	−at 4 months	+	+/−	+	NA	NA

m, male; f, female; +, present; −, absent; NA, not available; ∗, also cerebellar atrophy; †, death; +/−, moderate.

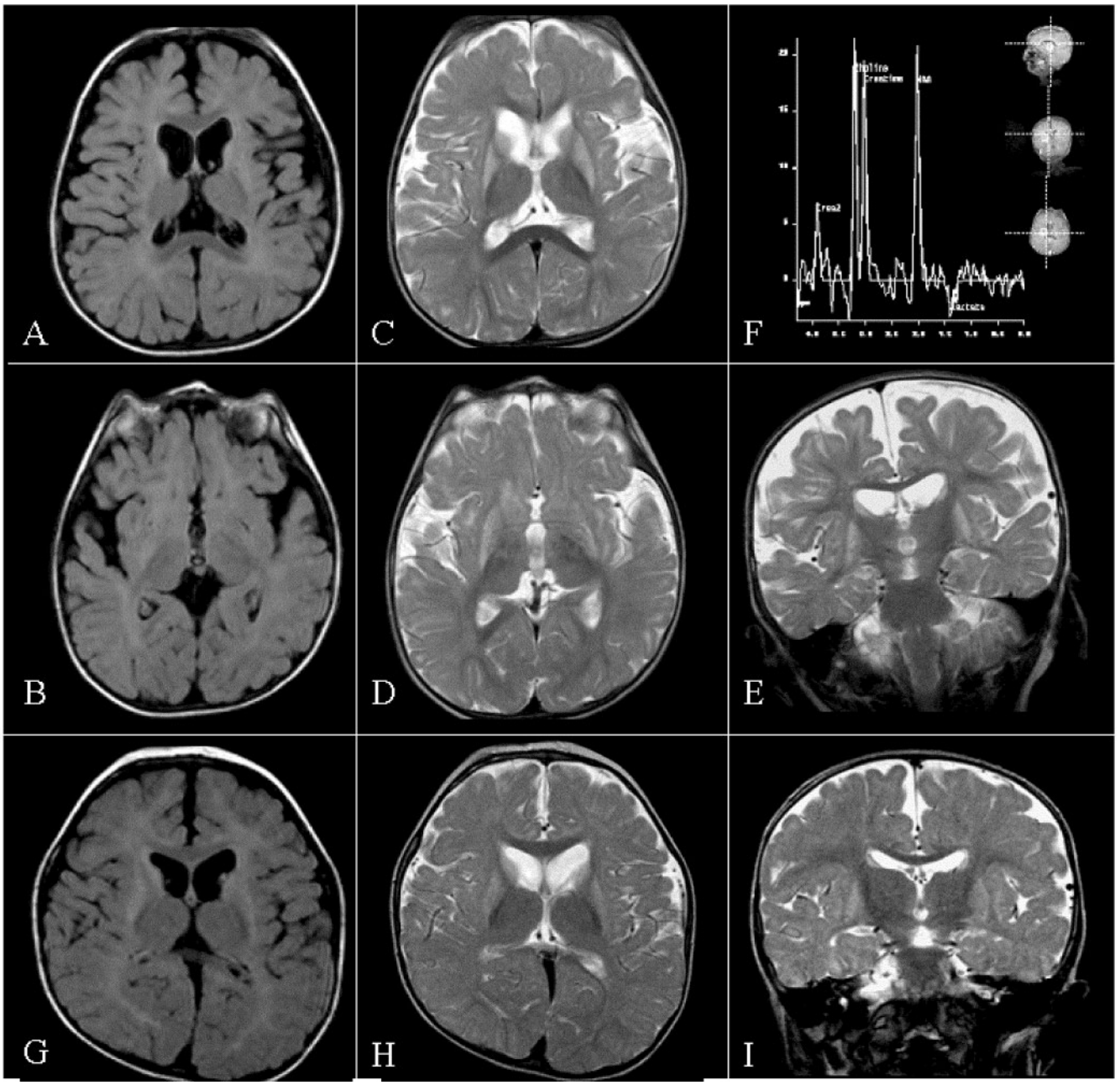

Fig. 3 MR images of patient 1 (**A**–**F**) and 2 (**G**–**I**). Axial fluid attenuated inversion recovery (FLAIR) (**A**, **B**, **G**,), axial (**C**, **D**, **H**,) and coronal (**E**, **I**) T_2-weighted images showing bilateral hyperintensity of putamen and caudate, enlargement of subarachnoidal spaces and of supratentorial ventricular system. ^{1}H-MRS of patient 1 (**F**), single voxel from right basal ganglia with TR 1500 ms and TE 135 showing an abnormal peak of lactate and decrease of NAA.

was detected; this mutation was present in the mother. The association between *SUCLA*2 and the clinical phenotype was further reinforced through simultaneous genotype analyses in the Faroese families. According to the selection criteria, six regions of 2 cM or larger and three additional regions with 10 or more homozygous SNPs were identified by whole genome scan in the Faroese patient group. In the region on chromosome 13 containing the *SUCLA*2 gene, 10 SNPs were homozygous covering 1.333 Mb (between SNPs rs724705 and rs1375662; 0.1 cM on the Marshfield map). In the affected cases from the Faroese families a homozygous splice site c.534 + 1G → A mutation was found in the *SUCLA*2 gene. DNA samples of all the parents (14 people) analysed by RFLP show heterozygosity for this mutation. Of the remaining 28 relatives analysed, 14 were heterozygous and 14 were homozygous for the wild-type. The gene has a relative common variation at position IVS3 + 9 (c > t). The c.534 + 1G → A mutation was always found to be associated with the polymorphism t. PCR analysis of cDNA did not show substantial levels of wild-type *SUCLA*2 cDNA

Table 2 Relevant body fluid metabolite data on the SUCLA2 patients and for comparison on a case with classical methylmalonic aciduria

Patient	1	2	3	9	10	11	12	Methylmalonic aciduria (MIM 251000)[d]	Reference
Plasma[a]									
Methylmalonate	1.3	2.4	NA	2.8	NA	3.2;4.1	3.9	1200–3300	<0.33
Lactate	4.0–6.0	7.5	3	4.0–8.5	2.1–3.4	2.1–5.0[c]	2.3–3.9[c]	1.2–2.3	0.5–1.7
Total carnitine	41	50	45	23	70	48	72	201–254	26–70
Free carnitine	26	26	24	12	26	30	40	50–68	20–55
C3-carnitine	1.4	3.0	NA	2.0	NA	4.2	7.5	80–133	0.23–0.92
C4-dicarboxylic carnitine	0.23	0.57	2.2	0.17	NA	0.39	0.50	2.1–2.6	<0.06
Urine[b]									
Methylmalonate	20	16	27	NA	60	80	80	6900–16 000	<5
C3-carnitine	1.1	1.0	NA	NA	NA	0.4	7.8	224	0.7–5.3[e]
C4-dicarboxylic carnitine	8.2	10	NA	NA	NA	17	13	16	0.04–0.5[e]

[a]Values are expressed in μmol/l except lactate in mmol/l; [b]values are expressed in μmol/mmol creatinine; [c]range of 4 values in a lactate day curve; [d]outside metabolic crisis ($n = 1$); [e]see reference by Mueller *et al.* (2003); NA, not available.

under our experimental conditions, although we cannot completely exclude the presence of a faint band at this position (Fig. 5). Two major fragments of a smaller size were amplified (Fig. 5, top). Sequence analysis of the 1.1 kb fragment showed skipping of exons 2, 3 and part of exon 4 (Fig. 5, bottom, B). The sequence of the lower band showed skipping of exons 2–4 (Fig. 5, bottom, C). Therefore, cDNA analysis was consistent with skipping of multiple exons, at least in cultured cells.

These three novel mutations of *SUCLA2* gene were not detected in over 200 control alleles.

In the remaining cases previously reported (Stromme *et al.*, 1995; Yano *et al.*, 2003; Deodato *et al.*, 2005), we did not detect *SUCLA2* mutations in the genomic DNA.

Frequency of the mutated allele on the Faroe Islands

Among 272 randomly selected anonymous neonatal DNA samples from the Faroe Islands we found nine heterozygotes and one homozygous case. Applying DNA fingerprint analysis using eight highly polymorphic short tandem repeat systems we could demonstrate that the homozygous sample shows a completely identical pattern to patient 12. This strongly suggests that it came from this individual. Thus, based on 11 mutated alleles among 544 investigated, allele frequency for the *SUCLA2* c.534 + 1G → A mutation in this population was calculated to be 2.0% [95% confidence interval(CI) 0.9–3.3%]. This predicts a carrier frequency of 1 : 25 (95% CI 1 : 15–55) and the theoretical frequency for homozygosity therefore amounts to 1 : 2500 (95% CI 1 : 910–11800).

Mitochondrial DNA depletion

The relative mtDNA/18S rRNA ratio was investigated in muscle and cultured fibroblasts (Fig. 6). The mtDNA content in muscle of patients 1 and 2 was reduced to 21 and 32% of the mean control value, respectively. The same patients had a reduction of 56 and 50% also in fibroblasts. Patient 12 showed normal mtDNA content in fibroblasts (muscle sample was not available for Southern blot analysis).

Discussion

Mild MMA, Leigh-like encephalomyopathy, dystonia and deafness characterizes a group of patients with *SUCLA2* gene mutations, in whom the key diagnostic features are represented by mild methylmalonic acid excretion combined with an abnormal profile of carnitine esters. Together with α-ketoglutarate dehydrogenase complex, succinate dehydrogenase and fumarate hydratase deficiencies, this condition also may be ranked among the inherited disorders of the TCA cycle (Pithukpakorn, 2005). Most of our patients presented with early-onset hypotonia, severe developmental delay or early neurological deterioration, slowly progressive dystonic/athetoid movements and neurosensorial deafness. These clinical signs and symptoms are fully in line with the description in the only family described with a SUCLA2 defect (Elpeleg *et al.*, 2005). Brain CT, MRI and post-mortem data demonstrate basal ganglia involvement closely resembling Leigh syndrome (Farina *et al.*, 2002) as often seen in several biochemically and genetically distinct mitochondrial encephalopathies (Zeviani and Di Donato, 2004). In the course of the first year of life, no abnormalities or mild cerebral atrophy with enlarged subarachnoideal spaces and widening of the ventricular system are observed. Later on basal ganglia involvement becomes obvious. At first, putamen and caudate seem to be specifically affected but later on also the other parts of the basal ganglia show abnormalities on the MRI. Unlike classical MMA (Trinh *et al.*, 2001), the globus pallidus can be spared or seems to be the

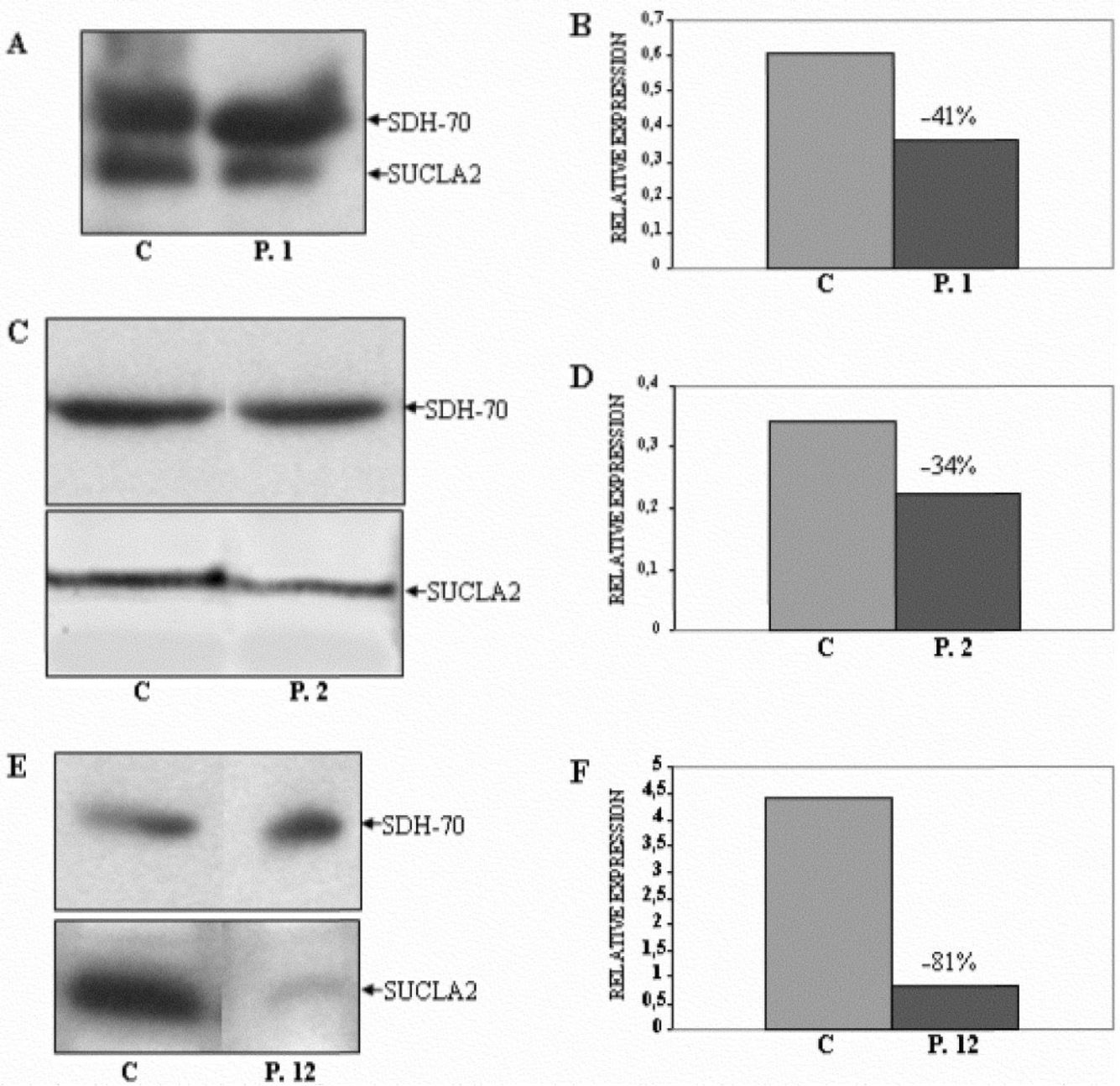

Fig. 4 Western blot (**A**, **C** and **E**) and densitometric analysis (**B**, **D** and **F**) of SUCLA2 in patient 1 (**A** and **B**), patient 2 (**C** and **D**) and patient 12 (**E** and **F**). Muscle mitochondrial protein (25 μg) was loaded in each lane. SUCLA2 content was normalized using the SDH-70 signal as a control for equal loading. Patients 1, 2 and 12 show a SUCLA2 reduction of 41, 34 and 81%, respectively. One experiment representative of three is reported.

least affected. In the end-stage of the disease, as observed post-mortem in one case, there was general basal ganglia involvement with only a very small residual volume of the nucleus caudatus remaining. These findings explain the dystonia in our patients and add defects in *SUCLA2* to the genetically and clinically heterogeneous group of dystonia syndromes (Tarsy and Simon, 2006).

The mild MMA, the abnormal excretion of some TCA cycle intermediates and the abnormal results of respiratory chain measurements pointed to *SUCLA2* as a possible candidate gene in our patients. Moreover their clinical features together with mtDNA depletion were all in line with the first description of a SUCLA2 deficiency by Elpeleg *et al.* (2005). The genome scan data on the Faroese families also confirmed our hypothesis. Accordingly, the three mutations that we subsequently detected in this gene have obvious features of pathogenicity. The p.Arg284Cys (found in three patients) and p.Gly118Arg mutations affect residues that are highly conserved during evolution (Johnson *et al.*, 1998). The c.534 + 1G → A affects the

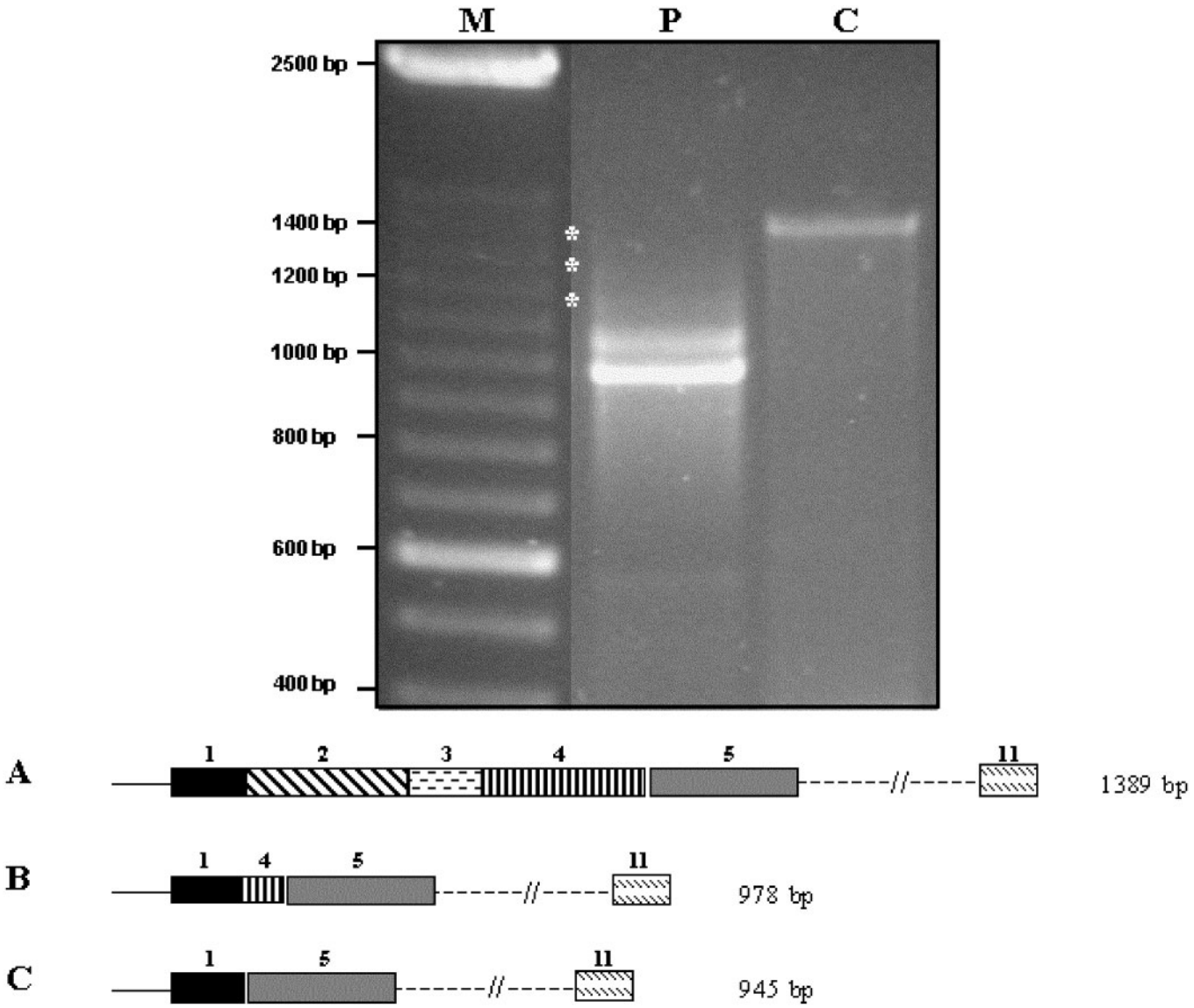

Fig. 5 Analysis of the c.534 + 1G → A mutation at the cDNA level in cultured fibroblasts of patient 12. *Top*: 1.2% agarose gel. *Bottom*: representative cartoon showing the effect of the mutation. At least two major transcripts, of 1.1 and 1 kb were identified: (**B**) one form skipping exons 2, 3 and part of exon 4; and another (**C**) skipping exons 2–4. The wild-type *SUCLA2* cDNA is also illustrated (**A**). M, DNA molecular marker size; P, patient 12; C, control; ∗, indicates additional bands likely corresponding to longer, less abundant *SUCLA2* mutant transcripts.

consensus splice site of intron 4 leading to multiple exon skipping. Interestingly, the p.Arg284Cys mutation was found heterozygous in both parents of patient 1 (DNA samples from relatives of patient 3 were not available), and in the father of patient 2, originating from two small villages in close proximity in southern Italy.

On the protein level we showed that the content of the *SUCLA2* gene product is diminished and that the enzymatic activity of SCS-A in muscle mitochondria in one case is reduced compared with those in mitochondria from control muscle samples. The residual activity might be due to the presence of additional, longer transcripts (see asterisks in Fig. 5, top panel). These functional data, together with absence of these mutations in a large set of control alleles and segregation of the mutation with the disease phenotype within families are strongly indicative for the pathogenic character of the novel variants identified in this study. The clinical spectrum of the SUCLA2 defect that emerges is rather homogeneous.

The *SUCLA2* gene encodes the β-subunit of the heterodimeric mitochondrial matrix enzyme SCS-A. GDP-forming succinyl-CoA synthase (SCS-G; EC 6.2.1.4) contains another β-subunit than SCS-A. SCS-A and SCS-G both may catalyse the reverse reaction from succinyl-CoA to succinate in the TCA cycle. Lambeth *et al.* (2004) have shown that mRNA expression as well as the amount of protein and enzymatic activity of SCS-A and SCS-G vary considerably between tissues in one species but also

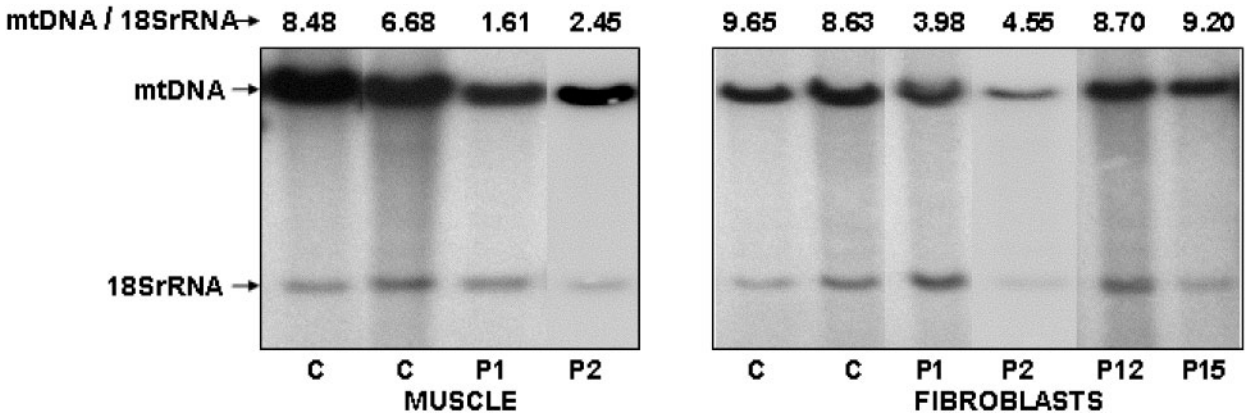

Fig. 6 Southern blot analysis of total DNA extracted from muscle tissue and cultured fibroblasts. Total DNA was digested with *Bam*H I and hybridized simultaneously with two probes: the total human mtDNA and a cloned fragment of the human nuclear I8S ribosomal gene (I8SrRNA). Individual relative ratios are shown at the top of the figure. Muscle mtDNA of patients I and 2 was reduced to 2I and 32% of the mean control value, respectively. The same patients showed a 56 and 50% reduction of mtDNAin fibroblasts. The mtDNA content in fibroblasts of patient I2 and I5 was normal. C, controls; P, patients.

between species. Western blot studies showed that the SCS-A β-subunit is highly expressed in testis, brain, heart and kidney while the SCS-G β-subunit occurs in high amounts in liver, kidney and heart but barely in brain and testis. The resulting deficiency of SCS-A activity in brain would, therefore, be the basis for the encephalopathy in the patients as the deficiency cannot be compensated in the brain by SCS-G activity. It has been observed that the β-subunit of SCS-G is much more highly expressed in anabolic tissues as kidney and liver and its role may be to support GDP-dependent anabolic processes (Lambeth *et al.*, 2004). At present it is unclear how the two enzymes function in concert within the same mitochondria and whether or not one enzyme may be able to compensate for the absence of the other.

The mild methylmalonic acidaemia with normal homocysteine, and the increased C4-dicarboxylic-carnitine level in plasma and even more in urine are the hallmarks of the disease at the metabolite level. As expected in a TCA cycle defect, variable lactic acidaemia and consistent lactate increase in CSF are accompanying characteristics in patients with a SUCLA2 defect. TCA cycle intermediates were found increased to a variable extent in the urine of the patients. The increase of methylmalonic acid in body fluids that is considerably less pronounced in patients with the SUCLA2 defect than in classical MMA (Deodato *et al.*, 2006), may be explained by the impaired conversion of succinyl-CoA to succinate, resulting in metabolite accumulation proximal to the enzymatic block (Fig. 1).

It may well be that the increased C4-dicarboxylic carnitine ester in our patients is a mixture of methylmalonylcarnitine and succinylcarnitine (Fig. 1). Succinylcarnitine is likely to be formed from accumulating succinyl-CoA. The concentration of C4-dicarboxylic carnitine is more increased in urine than in plasma. This may relate to the variable presence of SCS-A in different tissues and cell types.

The majority of the present population of the Faroe Islands descends from a small number of settlers. Thus, the genetic pool is small containing a number of mutations that occurred long ago. The observation that the pathogenic c.534 + 1G → A *SUCLA*2 mutation was always found associated with the *t*-variant in the polymorphism at position IVS3 + 9 is in line with a founder effect. Given the allele frequency of the *SUCLA*2 mutation on the island, it is estimated that 1 in 2500 neonates will be affected with the disease. A similar situation exists on the Faroe Islands for other genetic diseases including cystic fibrosis, glycogen storage disease type III and *N*-acetylglutamate synthase deficiency. It may be an option for the island to start a prevention project for such severe disorders comparable with the successful prevention projects for thalassaemia on Cyprus (Angastiniotis *et al.*, 1992; Angastiniotis, 1995) or the Dor Yeshorim prevention programme for Tay Sachs disease in a Jewish community in the US (Ekstein and Katzenstein, 2001). These programmes rely on premarital anonymous identification of heterozygotes to prevent affected births by identifying couples at risk. The allele frequency of the SUCLA2 defect on the island makes this disease a potential candidate for such an approach if it turns out that no rational therapy for this devastating disease can be found.

Our results indicate that the few reported 'unlabelled' cases of mild MMA may have variance in the phenotypic expression (Stromme *et al.*, 1995; Yano *et al.*, 2003; Deodato *et al.*, 2005) and are genetically heterogeneous. A few noteworthy differences were almost invariably observed between cases having changes in SUCLA2 and those who tested negative for gene mutations. These include the presence of sensorineural hearing deficit and the selective bilateral involvement of the putamen and caudate nucleus in patients with the SUCLA2 defect. The lack of common distinctive clinical, biochemical or molecular features in the

SUCLA2-negative patients suggests that further heterogeneity is to be expected.

The single report on *SUCLA2* mutations associates variations in this gene with a Leigh-like encephalomyopathy and mtDNA depletion (Elpeleg *et al.*, 2005). Our data confirm the mitochondrial DNA depletion in muscle tissue, as well as in some of the cultured fibroblasts. This confirms the previous finding that SCS-A activity may play a crucial role in controlling mtDNA maintenance (Elpeleg *et al.*, 2005). Moreover, the normal content of mtDNA that we observed in two out of four fibroblast samples may be due to the tissue specificity/heterogeneity of the genetic defect (Carrozzo *et al.*, 2003). The precise primary or secondary mechanism(s) responsible for mtDNA depletion, however, still remains unknown. Elpeleg *et al.* (2005) postulated that a disturbance in the tight complex of succinyl-CoA synthase with mitochondrial nucleoside diphosphate kinase may influence the phosphorylation of mitochondrial deoxyribonucleotide diphosphates resulting in diminished mitochondrial DNA synthesis. The SUCLA2 defect adds a further presentation to the already heterogeneous group of mitochondrial depletion syndromes.

In conclusion, we confirm and extend the findings on this inborn error of metabolism in the TCA cycle that must be carefully searched for by accurate metabolite analyses.

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