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A novel homozygous mutation in *SUCLA2* gene identified by exome sequencing

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ABSTRACT

Mitochondrial disorders with multiple mitochondrial respiratory chain (MRC) enzyme deficiency and depletion of mitochondrial DNA (mtDNA) are autosomal recessive conditions due to mutations in several nuclear genes necessary for proper mtDNA maintenance.
In this report, we describe two Italian siblings presenting with encephalomyopathy and mtDNA depletion in muscle. By whole exome-sequencing and prioritization of candidate genes, we identified a novel homozygous missense mutation in the *SUCLA2* gene in a highly conserved aminoacid residue. Although a recurrent mutation in the *SUCLA2* gene is relatively frequent in the Faroe Islands, mutations in other populations are extremely rare. In contrast with what has been reported in other patients, methyl-malonic aciduria, a biomarker for this genetic defect, was absent in our proband and very mildly elevated in her affected sister.
This report demonstrates that next-generation technologies, particularly exome-sequencing, are user friendly, powerful means for the identification of disease genes in genetically and clinically heterogeneous inherited conditions, such as mitochondrial disorders.

1. Introduction

Multiple mitochondrial respiratory chain (MRC) enzyme deficiency is a biochemical signature, common to a number of mitochondrial disorders, which can be due to diverse gene defects, including mitochondrial DNA (mtDNA) single or multiple deletions, mtDNA point mutations affecting mitochondrial tRNA genes, or mutations in nuclear genes related to several mitochondrial pathways, such as mtDNA maintenance and translation, assembly/regulation of respiratory chain subunits, biosynthesis of mitochondrial inner membrane phospholipids or MRC cofactors, and import of mitochondrial proteins [1]. In childhood, multiple MRC deficiency is often associated with mtDNA depletion, i.e., reduced mtDNA copy number; this condition, known as mtDNA depletion syndrome (MDS, OMIM ID: 251880) comprises a clinically and genetically heterogeneous group of autosomal recessive diseases [1,2]. Several nuclear genes have been associated with MDS. Mutations in the deoxyguanosine kinase (*DGUOK*) and thymidine kinase 2 (*TK2*) genes have been reported in the hepatocerebral [3] and myopathic [4] forms of MDS, respectively; and mutations in the catalytic subunit of the mtDNA polymerase gamma (*POLG1*) are associated with Alpers' syndrome, a condition characterized by mtDNA depletion in the brain and liver [2,5]. Less frequently, MDS can be caused by mutations in *MPV17*, encoding a small protein of unknown function embedded in the inner mitochondrial membrane [6], *RRM2B*, encoding the p53-inducible ribonucleotide reductase subunit 2, [7], *C10orf2*, encoding the mtDNA-Twinkle helicase [8], and *SUCLG1* [9] or *SUCLA2* [10], encoding the a and b subunits of the succinate-CoA ligase, (EC 6.2.1.5), a Krebs-cycle enzyme that catalyzes the reversible formation of succinate and ATP from succinyl-CoA and ADP. Defects in *SUCLA2* cause an encephalomyopathic MDS with moderate methylmalonic aciduria (OMIM ID: 612073). Mutations in this gene seem exceptionally rare, although a recurrent mutation has been reported in several patients originating from the Faroe Islands.

For several years, linkage analysis has been the most powerful and widely used strategy to identify the gene defects responsible for mendelian inherited disorders. However, this approach is very time consuming, and requires the availability of cohorts of homogeneous and informative families. Homozygosity mapping (e.g., by SNP arrays) is a potentiated version of linkage analysis, which had made possible the identification of more than 100 genes responsible for autosomal recessive diseases, but it requires the availability of informative, possibly

Abbreviations: MDS, mtDNA depletion syndrome; MMA, methylmalonic acid; MRC, mitochondrial respiratory chain; mtDNA, mitochondrial DNA; NGS, next-generation sequencing; OXPHOS, oxidative phosphorylation.

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large, consanguineous families. Nowadays, Next-Generation Sequencing (NGS) can be performed at affordable cost and timescale to analyze the coding regions (exome) of the human genome in single individuals or small families, including patients in which a clear genotype–phenotype correlation is absent or for clinically and genetically heterogeneous conditions, such as mitochondrial disorders. In addition, NGS-based homozygosity mapping can also be applied to select candidate genes and eventually find the causative mutation in patients from small, or even nuclear, consanguineous families [11,12].

Here we illustrate the power of NGS-based homozygosity mapping to identify a novel homozygous mutation in *SUCLA2* in a girl affected by severe, progressive encephalomyopathy with multiple MRC defects, but hardly any methylmalonic aciduria. A second, affected sister was then found to carry the same mutation.

2. Patients and methods

2.1. Patients

The index patient (PI) was an Italian girl born at term after normal pregnancy and delivery. Her parents were first cousins (Fig. 1A). Since the first months of life, the mother reported muscle hypotonia, failure to thrive, poor weight gain, and frequent vomiting. At 8 months of age the neurological examination showed normal cranial nerves, good visual contact but marked axial hypotonia with the absence of head control, in association with distal hypertonia, poor active movements, and brisk tendon reflexes. Nasogastric tube feeding was started because of severe dysphagia. Neurosensory hearing loss was documented by abnormal Brain Audiometry Evoked Potentials. Liver and kidney functions were normal. Biochemical exams revealed increased levels of lactate and pyruvate in both plasma (lactate 3057 μM, pyruvate 193 μM; normal values (nv): <2000 and <140) and CSF (lactate 2262 μM, pyruvate 144 μM; nv: <1800 and <120). Plasma carnitine short chain esters were moderately elevated (20 nM; nv: 3.4–10 nM). Methylmalonic acid (MMA) in urine was not tested, as well as the whole acylcarnitine profile. A brain MRI performed at 8 months showed bilateral abnormal signals in the caudate and putamina nuclei; these lesions persisted in a second brain MRI performed at age 24 months. The clinical features progressively worsened; at 18 months the patient showed a dystonic tetraparesis associated with bilateral ptosis and ophthalmoparesis, and severe cognitive impairment with no verbal development. She required nasogastric tube feeding until a percutaneous gastrostomy was performed at 7 years of age. Despite the severe clinical features she never presented metabolic crisis and the EEG was always normal. She developed multiple tendon retractions and progressive scoliosis since 10 years of age. The neurological condition slowly deteriorated; at her last examination, at 14 years of age, she showed normal growth parameters, severe ptosis with almost complete ophthalmoparesis, poor response to visual and tactile stimuli, diffuse muscle wasting, axial and limb hypotonia, areflexia, poor active movements and severe dystonic

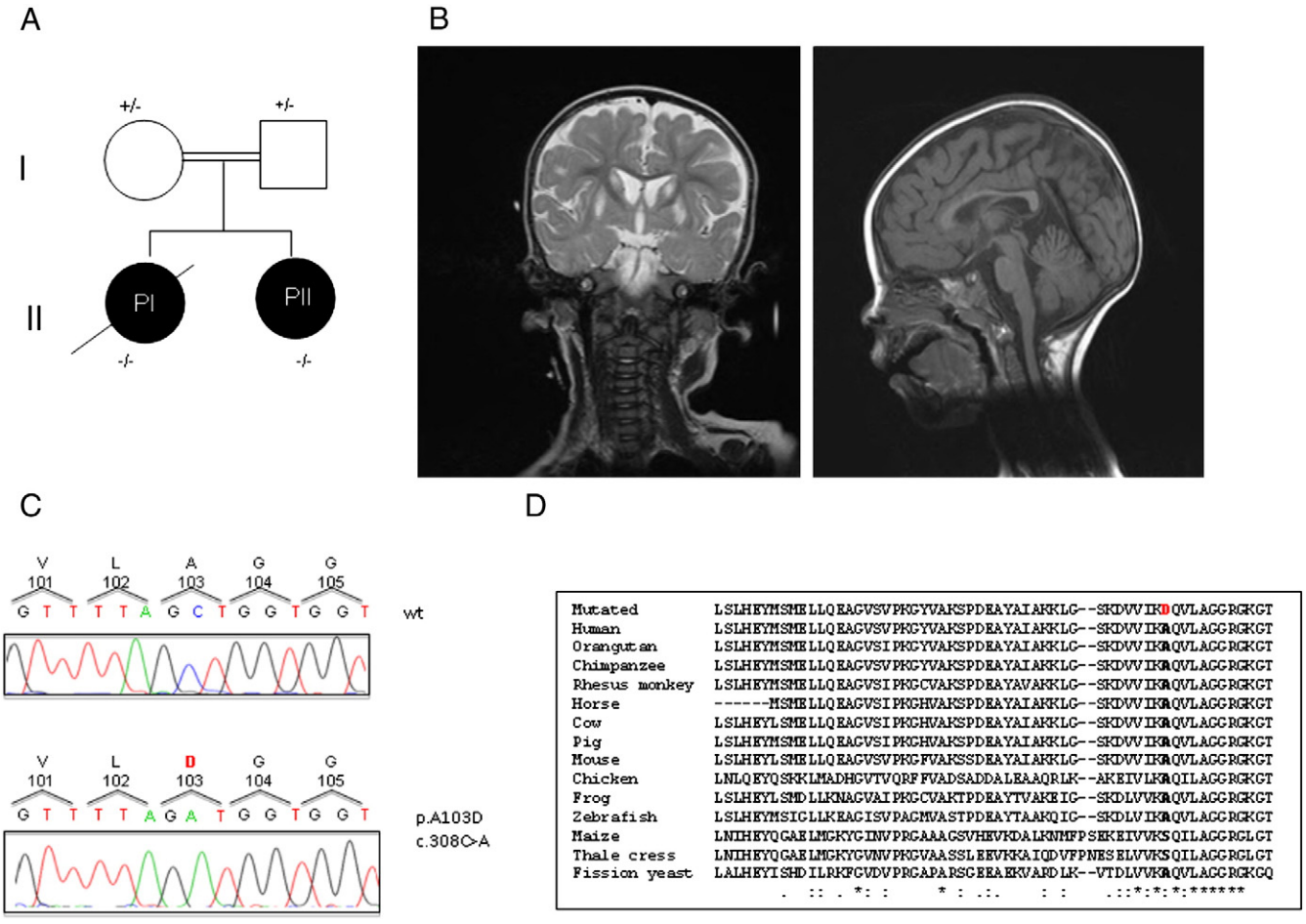

Fig. 1. A. Pedigree of the family. "−" corresponds to the allele with the Ala103Asp mutation; "+" corresponds to the wild-type allele. Black symbols indicate affected subjects. B. Brain MRI of patient II showing bilateral hyperintense lesions in caudate and putamina nuclei (*left panel*), and slight cerebellar and medullar atrophy (*right panel*). C. Electropherograms of the genomic region encompassing the c.308C>A substitution in a control wild-type subject (*upper panel*) and in patient I (*lower panel*). D. ClustalW multiple alignment of human SUCLA2 region containing the Ala103Asp mutation with aminoacid sequences from ortholog proteins: Orangutan (*Pongo pygmaeus*), Chimpanzee (*Pan troglodytes*), Rhesus monkey (*Macaca mulata*), Horse (*Equus caballus*), Cow (*Bos taurus*), Pig (*Sus scrofa*), Mouse (*Mus musculus*), Chicken Frog (*Xenopus laevis*), Zebrafish (*Danio rerio*), Maize (*Zea mays*), Thale cress (*Arabidopsis thaliana*), and Fission yeast (*Saccharomyces cerevisiae*).

posture with marked scoliosis. She died at 15 years of age due to respiratory failure during pneumonia.

Her 7-year younger sister (PII) showed a very similar clinical history. She was born from uneventful pregnancy and delivery, and presented with marked diffuse hypotonia since birth. She was examined at 2 months of age, showing axial hypotonia and limb hypertonia, brisk tendon reflexes and dystonic postures. Plasma lactate was 3500 μM, while the urinary MMA was in the normal range. During her first year of life she showed scarce growth improvement, frequent vomiting which required nasogastric tube feeding, hearing loss, and slowly progressive psychomotor regression. At 14 months of age, a brain MRI revealed the presence of severe bilateral abnormalities in caudate and putamen and slight alterations of dentate nuclei, with atrophy of cerebellum and medulla oblongata (Fig. 1B). The clinical evolution was similar to her older sister; at her last visit, at 5 years of age, she showed severe bilateral ptosis, incomplete ophthalmoparesis, spastic–dystonic tetraparesis with the absence of head control, scoliosis and marked irritability. No metabolic crises were reported.

2.2. Histochemical and biochemical studies

Informed consent was obtained from patient's parents, in agreement with the Declaration of Helsinki and approved by the Ethical Committees of the Foundation IRCCS Istituto Neurologico "C.Besta", Milan, Italy. Skeletal muscle biopsies from the left quadriceps were performed in both patients according to a standard protocol. 8 μm cryostatic cross sections were processed according to standard histological/histochemical techniques [13]. Succinate dehydrogenase (SDH) and cytochrome c oxidase (COX) activities were assessed as described [14].

Analysis of MRC activities was performed using standard spectrophotometric techniques [15] in both muscle homogenate and cultured skin fibroblasts.

2.3. Molecular studies

DNA was extracted from peripheral blood of the two patients and their parents using standard methods. For the analysis of mtDNA, total DNA was extracted from the muscle biopsies.

Southern blot analysis of muscle mtDNA and real-time (RT) PCR were used to quantify the mtDNA depletion, and sequencing of the entire mtDNA was performed, as described [16].

2.4. Whole exome sequencing

Whole exome sequencing was performed on genomic DNA obtained from skin fibroblasts of PII, in collaboration with "BGI-Shenzhen". The DNA sample was randomly processed by Covaris in fragments between 250 bp and 300 bp, and then adapters were ligated to both ends of the resulting fragments. For enrichment, the adapter-ligated template DNA was then amplified by ligation-mediated PCR, purified, and hybridized to the Nimblegen SeqCap EZ 44 M library. Then the captured library was loaded on Hiseq2000 platform for NGS. Illumina base-calling software 1.7 was used for base-calling with default parameters to process raw image files, and produced the sequences of each individual as 90 bp pair-end reads.

2.5. Data analysis

The sequenced reads were aligned to the human genome reference (UCSC hg 19 version, build37.1) using SOAPaligner2 [17]; SOAPsnp software [18] was used with default parameters to assemble the consensus sequence and call genotypes in target regions. The called variants were filtered in order to eliminate common variants (frequency >0.5% in dbSNP132, 1000 genomes or HapMap projects); changes in non-coding regions or resulting in synonymous substitutions were also removed. Next, the remaining non-synonymous/splice site (NS/SS) changes and microinsertions/deletions (INS/DEL) were prioritized according to (a) presence of homozygous mutations, based on recessive inherited trait and on the consanguinity of the parents, as indicated by the structure of our family; and (b) likely or ascertained mitochondrial localization of the corresponding protein, obtained by comparison with MitoCarta, the most complete inventory of mitochondrial proteins [19].

For the first point, exome-NGS data were used for homozygosity mapping, to detect homozygous regions in the genome. We selected SNPs and dbSNP-annotated genotypes as markers. To reduce the influence of sequencing errors, we required genotype quality of all markers to be ≥20: for heterozygous markers, we only selected those with covered ≥10 folds and with 30%–70% variation reads; for homozygosity markers, we only selected those with ≥5-fold covered reads, ≥95% of which were variation reads. Considering that some short exons would give misleading signals in homozygosity mapping, only long enough exons were selected: the minimum linked length was 1 Mb and the distance of adjacent exons no longer than 500 kb. Other exons were considered ambiguous and therein mutations reserved. We defined a region as homozygous if it was ≥1 Mb (≥5 Mb regions were considered more reliable) and contained at least one window of 500 markers, of which only ≤2 heterozygous, with a ≤500 kb distance between adjacent markers [12].

Additional indications to prioritize the candidate genes were obtained by using predictive software scoring the likelihood for pathogenicity (e.g. Polyphen, SIFT, Mutpred, Panther).

2.6. Validation of variants in candidate genes

PCR amplification of exons containing the prioritized variants and Sanger's sequencing (AbiPrism 3130XL) were performed in all family members in order to verify the data obtained by exome-NGS (primers and condition available upon request).

3. Results

Muscle histology was normal in both PI and PII: neither ragged red fibers (RRF) nor increase of lipid storage were noticed, but histochemically a partial diffuse reduction in COX activity was present in PII. Biochemical analysis revealed a multi-enzymatic defect of mtDNA-dependent MRC activities in muscle but not in fibroblasts from both patients (Table 1), whereas the activities of cII/SDH, the only MRC complex that has no mtDNA-encoded subunit, were normal. These data pointed to a defect of mtDNA maintenance or expression. The sequence of the entire mtDNA from skeletal muscle of PI and PII failed to show pathogenic mutations, but Southern-blot analysis did demonstrate partial reduction in the amount of mtDNA, with no deletions. Accordingly, real-time quantitative PCR showed that the amount of muscle mtDNA vs nuclear DNA was 57% for PI and 36% for PII, compared to age-matched control muscle samples.

By Next-Generation Sequencing, we generated 6.34 billion bases of sequence as paired-end, 90-bp reads, 91% of which was able to

Table 1
Biochemical analysis of OXPHOS activities in patient 1 (PI) and 2 (PII) muscle biopsies.

	PI	PII	CTR values
Complex I	**9.6**	**5.7**	13–24
Complex II	22.2	15.3	15–28
Complex III	**53**	**47.7**	88–167
Complex IV	**48**	**73.9**	120–280
Complex V	142	**107**	130–280
Citrate synthase[a]	197	105	80–210

In bold are reported values under control (CTR) range.
All enzymatic activities are normalized for Citrate Synthase activity.
[a] nmol/min mg.

Table 2
Candidate genes after filtering and prioritization steps.

Chr	Position	Ref	Gene name	Codons	Substitution	Pathogenicity prediction			
						Polyphen2	SIFT	Mutpred[a]	Panther[a]
chr13	48563080	G	SUCLA2	GCT=>GAT	A103D	Probably damaging	DAMAGING	0.811	0.87438
chr8	144398283	T	TOP1MT	ATA=>ATG	I448M	Benign	DAMAGING	0.544	0.36036

[a] For Mutpred and Panther, the probability of "deleteriousness" is reported (0 = not deleterious and 1 = highly deleterious). Chr: chromosome; Ref: reference nucleotide according to UCSC hg 19.

align to the human reference sequence. A total of 63.2% of these sequences mapped to the targeted exome region with 71.94-fold mean coverage. At this depth of coverage, more than 97% of the targeted bases were covered to pass our thresholds for variant calling. After identification of variants, we focused only on non-synonymous (NS) variants, splice acceptor and donor site mutations (SS), and short, frame shift coding insertions or deletions (indel) which were more likely to be pathogenic mutations, being located in homozygous regions. After filtering out variants present in public database and synonymous SNPs, 53 variants remained, including 6 splice-site mutations.

A total of 399 Mb regions were mapped as homozygous, which accounts for ~14% of the genome. The average and median length of homozygous regions is 1.71 and 1.33 Mb, respectively. After Exome Homozygosity mapping analysis, we obtained 25 variants in genes located in the homozygous regions, two of which code for mitochondrial proteins: *TOP1MT*, and *SUCLA2* (Table 2).

A T to C change, resulting in Ile448Met aminoacid substitution, was identified in *TOP1MT*, the gene encoding the mitochondrial topoisomerase I. The scores for pathogenicity obtained by *in-silico* analysis were discordant, ranging from "benign" for Polyphen, to "damaging" for SIFT software (Table 2). However, this variant was heterozygous not only in both parents but also in PI, which ruled out its causative role. Furthermore, a c.308C>A variant, that leads to a p.Ala103Asp substitution, was found in *SUCLA2* (NM_003850.2, NP_003841.1). The aminoacid change affects a highly conserved residue (Fig. 1C) and is predicted to be deleterious by using different software (Table 2). The variant was not present in our internal database composed of 844 exome sequences, and in the EVS database (http://evs.gs.washington.edu/EVS), containing >13000 alleles (about 8500 of European origin). The same mutation was identified in homozygosis in PI, whereas the parents were heterozygous. All these data, together with the clinical presentation of PI and PII, clearly indicate that *SUCLA2* mutation is the cause of the MDS in this family.

4. Discussion and conclusions

Maintenance of mtDNA is controlled by a homeostatic network, whose effectors are the various components of the mitochondrial replisome, the still largely unknown protein set that forms the mitochondrial nucleoid, and the many enzymes and carrier proteins that provide the mitochondrion with a balanced supply of deoxyribonucleotides, the mtDNA "building blocks". In principle, abnormalities in each of these players can cause MDS. Eight MDS genes are presently known, but their mutations account for only 60% of the cases. This gap prompted us to adopt exome NGS, an unbiased, systematic approach, to search for the responsible gene in our proband. This new technology carries out the time- and cost-effective sequence analysis of the coding regions of each and every gene. Increasingly powerful in silico filtering strategies are then available to prioritize candidate variants and eventually identify the causative gene. However, since we analyzed only one proband, the number of mutations obtained by exome NGS was still huge, even after the removal of common and synonymous variants, making the identification of the causative mutation a virtually unmanageable challenge. However, since the known consanguinity of the parents and the very low prevalence of the disease made the chance of two mutant alleles derived from distinct ancestors very low, we restricted the number of candidate genes to those contained within the regions of homozygosity obtained by the exome-NGS data. Using this method we identified a new mutation in *SUCLA2* gene. This gene encodes for the b subunit of succinyl-CoA ligase, a Krebs cycle enzyme with a yet unexplained role in mtDNA metabolism [2].

Mutations in *SUCLA2* have been reported in only 30 patients (Table 3), most of whom originating from a genetic isolate in the Faroe Islands that carry a homozygous G>A transition in intron 4, resulting in the skipping of exon 4 and predicting the synthesis of a truncated protein [20,21]. In 2005 Elpeleg et al. described for the first time a homozygous deletion/insertion (g.32720del43ins5) in *SUCLA2* associated with progressive encephalomyopathy and MDS. The affected members of this small Muslim family presented Leigh-like features with dystonic tetraparesis, alteration in the basal ganglia and deafness [10]. Subsequently, Ostergaard et al. described 12 patients born or originating from the Faroe Islands, presenting with encephalomyopathy, hearing loss and mild MMA. Interestingly, only one of these patients had abnormal MRI signals in the basal ganglia [21], but in another study, all 11 affected individuals from the Faroe Islands carrying the same homozygous mutation were hallmarked by Leigh-like lesions of the basal

Table 3
Laboratory and clinical features of *SUCLA2* patients.

Mutations	Homozygous c.534+1G>A	Homozygous c.850C>T	c.352G>A+c.850C>T	Homozygous c.789_802+29delinsATAAA	Homozygous c.308C>A
Protein	Splice mutation	p.Arg284Cys	p.Gly118Arg+p.Arg284Cys	Splice mutation	p.Asp103Ala
No. of patients	23	2	1	4	2
Origin	Faroe Islands	Italian	Italian/Romanian	Muslim	Italian
Hypotonia	Y	Y	Y	Y	Y
Dystonia	Y	Y	Y, moderate	Y	Y
Psycomotor delay	Y	Y	Y	Y	Y
Feeding problems	Y (91%)	Y	Y	nr	Y
Deafness	Y	Y	Y	Y	Y
Ophtalmoplegia/ptosis	Y (90%)	N	N	N	Y
CT/MRI	Cerebral atrophy (65%), BG (50%), cerebellar atrophy (25%)	Cerebral atrophy, BG	Cerebral atrophy, BG	BG, dilatation of the lateral ventricles	BG, cerebellar atrophy (50%)
MMA	Mild	Mild	Mild	nr	N/mild
Lactic acidosis	Y (78%)	Y	Y	Y	Y
mtDNA depletion	ms	ms, fbs	ms, fbs	ms	ms
References	[20], [21]	[20]	[20]	[10]	Present paper

Parentheses indicate percentages of patients presenting with the relative laboratory/clinical feature. BG: basal ganglia involvement; ms: skeletal muscle; fbs: fibroblasts; nr: not reported.

ganglia [20]. Three Italian families, each carrying different missense mutations of *SUCLA2*, presented with early onset encephalopathy with deafness and alterations in the basal ganglia. Other brain MRI features include cerebral and cerebellar atrophy [21]. Both our patients showed alterations in the basal ganglia and PII had also atrophy of cerebellum and a thin medulla oblongata. Similar to other *SUCLA2* mutant patients, ours showed severe dystonic tetraparesis with developmental delay and deafness. Feeding problems seem to be an invariant clinical feature of this disease, being present in almost all reported patients (Table 3). Our patients showed serious difficulties in feeding and became tube-fed very early in life. Severe and early onset ptosis and ophthalmoparesis were prominent features in our patents, and in the Faroe Islands patients as well [20], but were not reported in the other Italian patients. Methylmalonic aciduria, which was prominent in previous *SUCLA2* mutant patients, was absent or very mild in ours, which in fact prevented earlier diagnosis. The urinary MMA levels measured after the identification of the gene defect showed a modest elevation (value 11 μmol/mmol creatinine, nv: <5) only in PI.

Differently from the families reported by Ostergaard et al., the muscle morphology was normal in our patients, showing neither increased variability of fiber diameter, nor scattered hypertrophic fibers, nor lipid accumulation [21], whereas diffuse, partial COX deficiency was histochemically present, together with slight predominance of type I fibers. At the molecular level we identified an unreported mutation in exon 3, a c.308C>A nucleotide change producing an Ala to Asp substitution in position 103. The absence of this variant in SNP databases, the predicted deleterious effect of the substitution and the conservation of the mutant aminoacid residue strongly support the pathogenic role of the Asp103Ala change. Comparing the different mutations described, there is no clinical difference between Italian, Faroese and Muslim patients, suggesting that the corresponding mutations are functionally equivalent (Table 3).

MDS ranges from isolated myopathy to fatal liver failure or multisystem involvement [2]. However, since most of the causing genes are ubiquitously expressed, the tissue specificity of mtDNA depletion remains unexplained. Encephalomyopathic MDS is typically associated with mutations in *SUCLG1* (also reported as *SUCLA1*) or in *SUCLA2*, coding for subunits of succinyl-CoA ligase, a heterodimeric enzyme, composed of an invariant alpha subunit (encoded by *SUCLG1*) and a beta subunit (encoded by *SUCLG2* or by *SUCLA2*) that determines whether the enzyme is GTP- (G-SUCL) or ATP-dependent (A-SUCL) [22]. Thus, the two enzymes, G-SUCL and A-SUCL, catalyze a similar reaction by using different phosphate donors.

The tissue specificity of *SUCLA2* mutations can be partly due to the fact that the A-SUCL predominates in the brain and skeletal muscle [23], whereas G-SUCL is predominantly expressed in the liver. This different tissue distribution of the two isoforms is likely to prevent the normal G-SUCL from fully compensating a crippled A-SUCL in critical tissues, namely the brain and skeletal muscle [24].

The link between impairment of SUCL, a Krebs' cycle enzyme, and mtDNA depletion is unclear; a defect in dNTP supply has been suggested, since both SUCL isoforms are physically associated with the nucleoside diphosphate kinase (NDPK), an enzyme involved in the dNTP salvage pathway during mtDNA synthesis [2,10]. A recent work in cellular models has confirmed the fundamental role of SUCL (G-SUCL>A-SUCL) in the maintenance of mtDNA, by modulating NDPK activity [24].

Inherited diseases due to *SUCLA2* mutations, as well as many other mitochondrial disorders, are rare and clinically heterogeneous conditions. Our report underlies the difficulty to set up a molecular diagnostic procedure in these disorders. In fact, even if some biochemical and clinical features are suggestive for peculiar genetic forms, such as MMA in *SUCLA2*- associated MDS (MDS5), these biomarkers can occasionally be normal or overlooked. NGS is a powerful and unbiased procedure, particularly suitable for mitochondrial disorders, which are characterized by high genetic and clinical heterogeneity and relatively loose genotype/phenotype correlation.

Conflict of interest

All authors declare that they have no conflicts of interest.

Web resources

The URLs for data presented herein are as follows:

Exome Variant Server (EVS), http://evs.gs.washington.edu/EVS
MutPred, http://mutpred.mutdb.org
Online Mendelian Inheritance in Man (OMIM), http://www.omim.org
Panther, http://www.pantherdb.org
Polyphen2, http://genetics.bwh.harvard.edu/pph2
Sorting Intolerant From Tolerant (SIFT), http://sift.bii.a-star.edu.sg

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