ORIGINAL ARTICLE

Mutations in the mitochondrial cysteinyl-tRNA synthase gene, *CARS2*, lead to a severe epileptic encephalopathy and complex movement disorder

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ABSTRACT

Background Mitochondrial disease is often suspected in cases of severe epileptic encephalopathy especially when a complex movement disorder, liver involvement and progressive developmental regression are present. Although mutations in either mitochondrial DNA or *POLG* are often present, other nuclear defects in mitochondrial DNA replication and protein translation have been associated with a severe epileptic encephalopathy.

Methods and results We identified a proband with an epileptic encephalopathy, complex movement disorder and a combined mitochondrial respiratory chain enzyme deficiency. The child presented with neurological regression, complex movement disorder and intractable seizures. A combined deficiency of mitochondrial complexes I, III and IV was noted in liver tissue, along with increased mitochondrial DNA content in skeletal muscle. Incomplete assembly of complex V, using blue native polyacrylamide gel electrophoretic analysis and complex I, using western blotting, suggested a disorder of mitochondrial transcription or translation. Exome sequencing identified compound heterozygous mutations in *CARS2*, a mitochondrial aminoacyl-tRNA synthetase. Both mutations affect highly conserved amino acids located within the functional ligase domain of the cysteinyl-tRNA synthase. A specific decrease in the amount of charged mt-tRNA^{Cys} was detected in patient fibroblasts compared with controls. Retroviral transfection of the wild-type *CARS2* into patient skin fibroblasts led to the correction of the incomplete assembly of complex V, providing functional evidence for the role of *CARS2* mutations in disease aetiology.

Conclusions Our findings indicate that mutations in *CARS2* result in a mitochondrial translational defect as seen in individuals with mitochondrial epileptic encephalopathy.

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INTRODUCTION

Mitochondrial dysfunction is a common cause of human disease with an incidence of 1:5000 to 1:10 000 live births.[1] Despite the high frequency of disease, a majority of patients remain without a genetic diagnosis. Mitochondrial disease has been associated with mutations in 13 mitochondrial-encoded genes and in >200 nuclear-encoded genes.[2] Furthermore, there are >1000 nuclear genes that encode mitochondrial-targeted proteins, providing a large number of probable candidate genes as the cause of mitochondrial dysfunction.[3] The large number of candidate genes and heterogeneous manifestations of disease are the main impediment to the genetic diagnosis. However, recent developments in high-throughput sequencing have significantly increased the detection of mutations in nuclear-encoded genes associated with mitochondrial disease.[4–6]

Despite the increased utility of genetic testing, providing proof of pathogenicity of novel variants remains challenging and mitochondrial functional studies remain an integral part of the evaluation. The results of functional studies may help narrow down potential candidate genes to a specific gene function as well as provide a means to validate genetic results. Decreased activity of respiratory chain enzymes or altered oxidative phosphorylation coupling can illustrate mitochondrial dysfunction. Isolated deficiency of the activity of a single respiratory chain enzyme is typically the result of genetic alterations in structural subunits or assembly proteins for respiratory chain complexes. Combined deficient activity of multiple respiratory chain enzymes may be caused by defects in proteins required for mitochondrial DNA (mtDNA) maintenance, transcription or translation.

Although the clinical findings in mitochondrial disease are heterogeneous, central nervous system involvement is common, often manifesting as neurological regression or movement disorders. Various epileptic conditions have been well described in patients with mitochondrial disease.[7–9] Severe mitochondrial epileptic encephalopathies are most commonly associated with myoclonic epilepsy with ragged red fibres and Alpers' syndrome. Patients with Alpers' syndrome often present in infancy with cortical atrophy and prominent epilepsy, which may evolve into intractable epilepsy,[10 11] and a deficiency of respiratory chain enzymes is prominently present in liver, whereas other tissues, such as skeletal muscle, can be normal.[12] Mutations in the polymerase-γ gene, *POLG*, account for the majority of cases of Alpers' syndrome.[13 14] Other genes involved in mitochondrial replication and translation have also been associated with mitochondrial epileptic encephalopathies.

We describe a child with a severe epileptic encephalopathy, complex movement disorder and a combined respiratory chain enzyme deficiency in whom we identified mutations in a gene involved in mitochondrial translation. Multiple mitochondrial respiratory chain enzyme deficiencies in liver and decreased synthesis of mtDNA-encoded subunits were consistent with a defect in either mitochondrial transcription or translation. Using trio-based exome sequencing, we identified compound heterozygous mutations in *CARS2*, a mitochondrial cysteine-specific aminoacyl-tRNA synthetase, consistent with the patient's phenotype and the mitochondrial respiratory chain enzyme analysis.

Subject

The infant, the second child of non-consanguineous parents of Scandinavian ethnicity, presented at 5 weeks of age with episodes of opisthotonus and feeding difficulties resulting in failure to thrive for which he received a gastrostomy tube and Nissen fundoplication. His early clinical course was marked for a delay in acquiring developmental milestones, and he developed hypotonia and abnormal movements. His highest level of developmental function included ambulation with the assistance of a walker, hitting a toy and babbling. He declined in function over a 3-year period with regression usually associated with infections and resulting in the loss of skills such as rolling over, reaching and grasping. He developed a complex movement disorder with chorea, dystonia including oculogyric episodes, myoclonus, startle myoclonus and developed microcephaly.

At 3 year 10 months, he developed medically refractory complex partial status epilepticus, with severe background slowing, multifocal epileptiform discharges, frequent focal myoclonic and complex partial seizures. These seizures were unresponsive to midazolam and pentobarbital infusions, the ketogenic diet and multiple antiseizure medications. He was discharged in complex partial status epilepticus and has remained in this condition for >5 years. Myoclonus remained frequent, but his chorea and dystonia resolved. He had central and obstructive sleep apnoea and swallowing dysfunction. He developed osteoporosis and presented spontaneous fractures of both humeri at age 6 years. Presently, he is alert, has frequent myoclonus of his distal limbs or mouth, is severely hypotonic and does not have antigravity movements of his limbs or head. He is areflexic and has no purposeful or elicited eye movements on oculocephalic manoeuvre. He communicates with family members using an augmented communication device by moving a finger or foot to activate a button. He receives a very low caloric diet via G-tube to avoid excessive weight gain.

Brain MRI showed progressive atrophy of the cortex and, more prominently, of the white matter with focal increased T2 signal in cortex and white matter, a very thin corpus callosum and atrophic cerebellar vermis (figure 1). He developed multifocal epileptiform discharges on EEG most prominent over the posterior quadrant, with slowing posteriorly and, at present, continuous multifocal high-amplitude epileptic discharges on EEG. Serum lactate was increased in 50% of samples obtained, up to 5.8 mM maximum. Lactate in spinal fluid was increased at 2.6–3 mM with a normal lactate/pyruvate ratio of 12 and 13, respectively. Serum alanine was mildly elevated at 511 μM and cerebrospinal fluid (CSF) alanine was mildly elevated at 45 and 52 μM (normal range 13–48 μM). His albumin has been low frequently, and almost continually since age 6 years (average of 3.1 g/dL, age-related normal range 3.7–5.6 g/dL). The coenzyme Q level in serum was normal (0.67 mg/L, normal range 0.4–1.19 mg/L). Other liver function tests such as bilirubin, transaminases, ammonia and clotting factors have been normal and renal function has been normal including absence of proteinuria. Biopsies for mitochondrial evaluation in skeletal muscle, liver and skin were obtained at 3 year 10 months. The liver showed mild microvesicular steatosis with normal mitochondrial ultrastructure, and the skeletal muscle had normal histology. He had normal enzyme activities for biotinidase and pyruvate dehydrogenase, and normal molecular investigations for mitochondrial bioenergetic disorders included mtDNA point mutations and deletions in leucocytes, complete mtDNA sequencing in skeletal muscle and sequencing of *POLG1*, *TK2*, *ANT1*, *DGUOK*, *SUCLA2*, *POLG2*, *MPV17*, *TWINKLE (C10orf2)*, *RRM2B* and *TIMM8A*. His karyotype was normal, and array-based comparative genomic hybridisation was negative for clinically significant copy number variations.

METHODS

Respiratory chain enzyme analysis

Respiratory chain enzyme activities for complexes I, II, II+III, III, IV and citrate synthase (CS) were assayed in post 600 g supernatants spectrophotometrically on a Cary 300 spectrophotometer at 30°C in cultured skin fibroblasts and in skeletal muscle as described with some modifications.[15 16] For complexes I, II, II+III and CS, enzyme activities were calculated as initial rates (nmol/min), and for complexes III and IV as first-order rate constants. All activities were normalised to the total protein content in each sample and expressed as ratios over the activity of CS and of complex II. The natural log of the activities and of the ratios in control samples was normally distributed, and the results were thus expressed as Z-scores. The

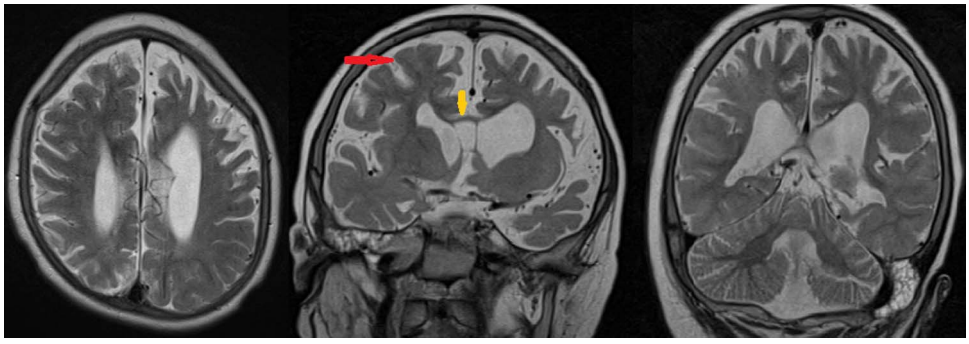

Figure 1 Brain MRI. MRI of the brain at age 6 years showing axial and coronal images on a T2-weighted sequence. Global atrophy of the cerebrum and the cerebellar vermis with secondary ex vacuo dilatation of the lateral and third ventricle is seen. The cerebral white matter shows atrophy. A red arrow shows a deep sulcus representative of the cerebral atrophy, and the orange arrow illustrates the very thin corpus callosum.

normal ranges were derived from 25 muscle controls, 16 liver controls and 20 control fibroblasts.

The integrity of the assembly of complexes I, II, IV and V was evaluated using blue native polyacrylamide gel electrophoretic (BN-PAGE) analysis followed by in-gel activity stain in skeletal muscle and in a cultured skin fibroblast pellet using a previously published method.[17 18] This method can identify defects in mitochondrial transcription and translation by showing bands of incompletely assembled complex V as reported earlier.[19]

DNA extraction

High-quality, unfragmented genomic DNA (A260/A280≥1.8 and A260/A230≥1.9) was extracted from whole blood obtained from the subject and his parents using a FujiFilm DNA Extraction Robot, from FujiFilm Medical Systems (Stamford, Connecticut, USA) using QuickGeneR DNA whole blood kit L, from Kurabo Industries (Osaka, Japan), and using the salting out method from QIAGEN following the manufacturer's instructions.

Exome sequencing and data analysis

Exome sequencing on the subject and his parents was performed using Nimblegen 44Mb SeqCap EZ Exome V.2.0 (Roche Nimblegen, Madison, Wisconsin, USA) followed by sequencing on an Illumina HiSeq 2000 (Illumina, San Diego, California, USA). Approximately 50 million, 90 bp, paired-end reads were obtained from each individual sequenced. On average, over 50× coverage was obtained from the subject and his parents. Sequence reads were analysed using well-established mapping and variant detection software (details provided in online supplementary methods). The sequence data from the patient and parents were used to test for causal variants as previously described.[20]

PCR and Sanger sequencing

Variants identified in *CARS2* (NM_024537.2, ENST00000 257347) were further validated by Sanger sequencing in the subject and his parents. Primers were designed to amplify and sequence exons 6 and 7 of *CARS2* where the identified sequence variants are located in the proband. Primers were designed to amplify and sequence all exon and intron boundaries of *CARS2* in the additional 15 subjects. Genomic DNA (100 ng) was amplified using PCR, with reactions and conditions as follows: Promega GoTaq Hot Start' kit (Promega, Madison, Wisconsin, USA) with 1× Master Mix and 400 nM of each primer. The PCR began with an initial cycle at 95°C for 3 min, followed by 30 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 1 min, finishing with extension at 72°C for 5 min. Amplified PCR products were sequenced using the PCR primers as sequencing primers on an ABI (Life Technologies, Carsbad, California, USA) PRISM *3730xl* at a commercial Clinical Laboratory Improvement Amendments (CLIA)-certified sequencing facility.

Protein analysis

For SDS-PAGE, 5 μg of protein was separated on a 5–20% gradient polyacrylamide gel with sodium dodecyl sulfate followed by western blotting, and detection using the appropriate primary antibody, followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibody, and visualised by chemiluminescence. Assembly of mitochondrial complexes was further evaluated by western blotting after BN-PAGE of 10 μg of mitochondrial protein, and probed with an antibody against the complex I subunit NDUFS2 (Mitosciences, MS114), a subunit that incorporates into the early stages of complex I assembly. The full-length amino acid sequences of human CARS2 and the *Escherichia coli* cysteinyl-tRNA syntethase-$tRNA^{Cys}$ complex were aligned and the relative positions of homologous residues were modelled using the crystal structure of the *E. coli* enzyme (1U0B).[21]

Aminoacylation assay

Aminoacylation of mt-tRNA-Cys was analysed as described previously.[22] Briefly, total RNA was extracted from fibroblasts using Trizol reagent (Life Technologies) following the manufacturer's instructions, with the final pellet resuspended in 10 mM NaOAc at pH 5.0 and kept at 4°C to preserve the aminoacylation state. For the deacylated control, the pellet was resuspended in 200 mM Tris-HCl at pH 9.5 and incubated at 75°C for 5 min, followed by RNA precipitation and resuspension in 10 mM NaOAc at pH 5. Next, 15 μg of RNA was separated on a 6.5% polyacrylamide gel (19:1 acrylamide:bisacrylamide) containing 8 M urea in 0.1 M NaOAc pH 5.0 at 4°C and electroblotted (Bio-Rad, Trans-Blot Cell) onto a nylon transfer membrane (Hybond, GE). Following UV-crosslinking, the membrane was hybridised with appropriate radiolabelled riboprobes, washed and imaged using a PhosphorImager.

Correction studies

The full-length *CARS2* cDNA in vector pOTB7 was obtained from Open Biosystems (Catalog MHS1011-60885 Accession (genbank): BC007220). The cDNA fragment was excised using *Eco*RI and *Xho*I and subcloned into the pCHAC-MCS-IRES-mWasabi vector of the Gryphon retrovirus expression system, which contains the 5′LTR, the packaging signal and the 3′LTR with the gene inserted in the multicloning site followed by an IRES and a mWasabiGFP protein (Allele Biotechnology, San Diego, California, USA). The vector was transfected into the Gryphon amphotrophic packaging cells by electroporation using the Amaxa Nucleofector II electroporation system (Lonza Group, Basel, Switzerland). The resulting viral supernatant used for transduction of the patient cultured skin fibroblasts contained 4 μg/μL polybrene. More than 75% of fibroblasts showed active fluorescence from the WasabiGFP showing good transfection efficiency. The effect of the introduction of the native *CARS2* cDNA construct was followed by BN-PAGE gel analysis.

Additional patient samples

To identify the frequency of CARS2 mutations in patients with mitochondrial translation defects, samples from 15 other patients were identified. These patients each had a combined respiratory chain enzyme deficiency, fragmented complex V on BN-PAGE analysis and normal mtDNA analysis, thus indicating a defect in either mitochondrial transcription or translation.[19] Primers were designed to amplify and sequence all exons and exon–intron boundaries of *CARS2* and subjects were Sanger sequenced after PCR amplification.

RESULTS

Respiratory chain enzyme analysis

We observed a combined deficiency of the activities of complex I and III and low activity of complex IV (not statistically significant) in the liver of the subject (see online supplementary table S1). In muscle, there was a tendency towards low activity for complexes I and IV, which did not reach statistical significance. This may be due to a compensatory amplification of mtDNA as a result of mitochondrial dysfunction. This is supported by the significant elevation of mtDNA copy number in muscle at 315%

of age and tissue-matched controls (value: 5506; control: 1746 ±361). In fibroblasts, complex IV activity tended to be low as well. The BN-PAGE gel analysis with in-gel activity staining showed the presence of additional bands reflecting incomplete assembly of complex V present in both skeletal muscle and cultured skin fibroblasts (figure 2A, B). Such bands of lower molecular mass in complex V have been shown to occur in patients with defects in complex V assembly, with mtDNA depletion, or in defects in mitochondrial transcription or translation.[19] The combined deficiency in liver indicates that the deficiency is not limited to complex V assembly, and a western blot with an antibody against NDUFS2 indicated incomplete assembly of complex I (see online supplementary figure S1). The high mtDNA copy number in skeletal muscle excludes mtDNA depletion syndrome, thus indicating that the defect affects mitochondrial transcription, RNA processing or translation. A large number of genes are involved in mitochondrial RNA transcription and processing and in the mitochondrial translation process, thus making an exome analysis approach preferable.[23–29]

Exome sequencing

Exome sequencing was performed in the proband and the unaffected parents. The downstream analysis was focused on non-synonymous coding variants, coding InDels and variants affecting splice sites. We first filtered out common variants present in dbSNP and 1000 Genomes data. Parental exome sequencing data were used to identify possible pathogenic variants under various inheritance models including dominant (de novo mutations) and recessive (compound heterozygous, homozygous and X-linked hemizygous mutations) models. This resulted in the identification of three candidate genes, *DMXL1*, *CCDC120* and *CARS2*, which were considered for further analysis (see online supplementary table S2).

Using a recessive inheritance model, we identified two heterozygous, non-synonymous sequence variants in the mitochondrial cysteinyl-tRNA synthetase, *CARS2*, that were considered potentially pathogenic. The patient inherited an in-frame deletion c.649_651delGAG (p.Glu217del) from his father and a missense mutation c.752C>T (p.Pro251Leu) from his mother in *CARS2*. Sanger sequencing confirmed the presence of both the mutations in the patient and inheritance of one mutation from each parent, respectively. Both mutations affect highly conserved residues (figure 3) in the cysteinyl-tRNA synthetase located within the conserved functional ligase domain (Cys-tRNA/MSH-ligase). The missense mutation Pro251Leu is predicted deleterious by bioinformatics software (Polyphen2, score 1.0). The Pro251 missense mutation was modelled on a homologous structure from *E. coli* (1U0B). The deleted amino acid Glu297 could not be modelled because it is located in a nine amino acid region that has no homology in the bacterial enzyme. Pro251 is located in the CP domain near several important residues including His254, which is involved in hydrogen bonding with RNA phosphates; Trp253, which is involved in tRNA^{Cys} substrate binding; and a conserved group of four hydrophobic amino acids (Ile169, Trp242, Thr166, Val121) neighbouring Pro251 in the 3D structure.[21] This suggests that the mutation causes a structural change in an important region of CARS2 (see online supplementary results, figure S2 and S3).

CARS2 (NM_024537.2) contains 15 exons (transcript length 1.88 kb) encoding a mitochondrial-targeted (62 AA mitochondria-targeting peptide) protein with 564 amino acid residues (NP_078813.1; CCDS9514.1). At the time of analysis, defects in *CARS2* had not been reported or associated with a specific phenotype. However, this finding was consistent with the pathophysiology of other reported aminoacyl-tRNA synthetase (ARS) defects, where mutations are mostly clustered in conserved regions of the core catalytic domain and the tRNA anticodon-binding domain.[30] Thus, *CARS2* was considered the best candidate for a role in disease aetiology prompting further investigation.

Mitochondrial tRNA aminoacylation analysis

Functional impact of the *CARS2* mutations on the mitochondrial cysteinyl-tRNA was assessed by high-resolution northern blotting of RNA isolated from patient primary skin fibroblasts. The use of low pH throughout the procedure allows for distinction

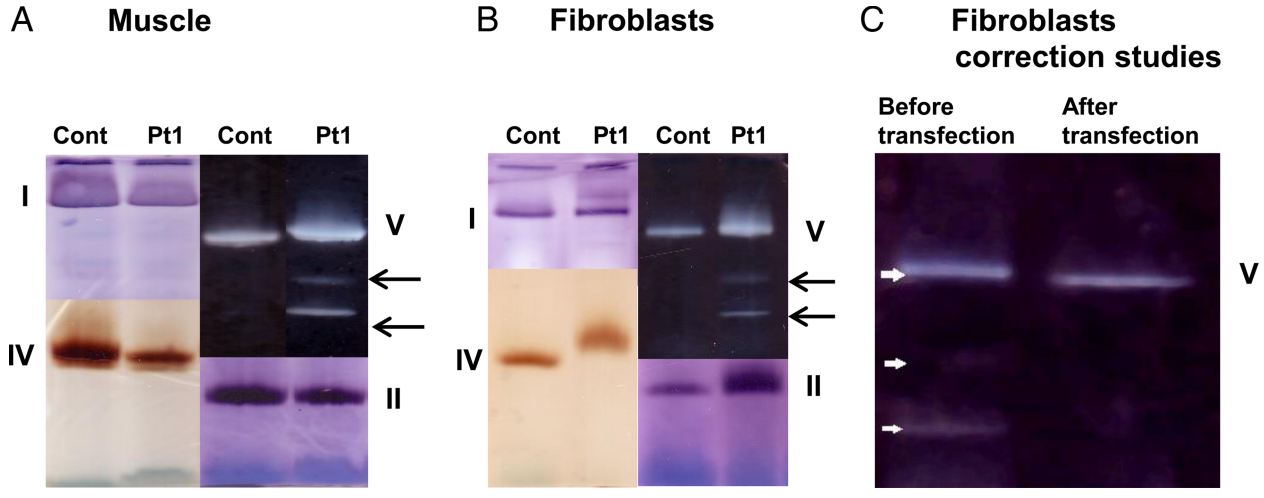

Figure 2 Characterisation of biochemical and molecular defects in subject cells and correction studies. Blue native polyacrylamide gel analysis of mitochondrial respiratory chain complexes I–V with in-gel activity staining, assayed in skeletal muscle (A) and cultured skin fibroblasts (B) from the proband compared with a healthy control. In both tissues from the proband, bands with a lower molecular mass were seen following staining for complex V indicating the presence of incompletely assembled complex V. (C) Correction studies by transfection of wild-type *CARS2* into patient-cultured skin fibroblasts. Before transfection, an additional lower band and a faint middle band are seen, representing incomplete assembly of complex V. After transfection of the cultured skin fibroblasts with the wild-type *CARS2* cDNA, these additional bands representing incompletely assembled complex V were no longer present.

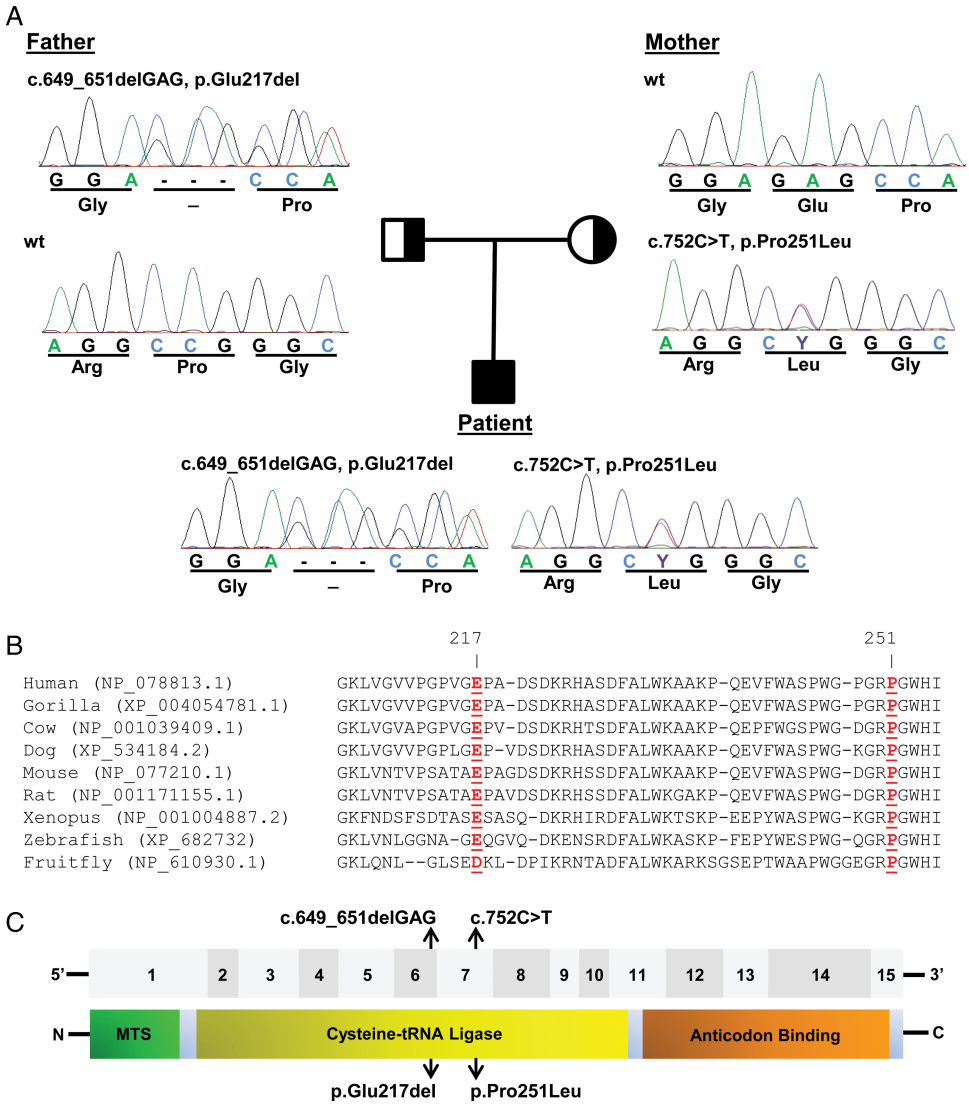

Figure 3 Pathogenic variants in *CARS2*. (A) Partial chromatograms showing Sanger sequencing results of *CARS2* in the patient and his parents. The patient has an in-frame deletion (c.649_651delGAG, p.Glu217del) inherited from his father and a missense mutation (c.752C>T, p.Pro251Leu) inherited from his mother. (B) Comparative analysis of CARS2 proteins from multiple species demonstrated that Glu217 and Pro251 (highlighted in red) are evolutionarily conserved. Orthologs were identified by using BLASTP, and the alignments were performed by using ClustalW. Protein accession numbers are in parentheses. (C) Top panel shows the 15 exons of *CARS2* cDNA as grey boxes. Bottom panel shows the CARS2 protein domains, including mitochondrial targeting sequence (MTS), the catalytic cysteine-tRNA ligase domain and the anticodon binding domain. Locations of variants are indicated by arrows. Paternal in-frame deletion is located in exon 6 and maternal missense mutation is located in exon 7. Both variants are located within the ligase domain of CARS2 protein. WT, wild-type.

between the aminoacyl-tRNA and the uncharged tRNA. A decrease of the ratio between the aminoacylated and deacylated form of mt-tRNA^{Cys} was detected, whereas the aminoacylation of control mt-tRNAs, tRNA^{His} and tRNA^{LeuUUR} was normal (figure 4). Additionally, the steady-state levels of aminoacylated tRNA^{Cys} were normal in the patient-derived sample compared with the controls (figure 4).

Protein analysis and correction studies

Western blot analysis was performed in cultured skin fibroblasts to analyse the effect of the *CARS2* mutations on CARS2 protein levels. CARS2 was significantly reduced in the patient compared with controls (see online supplementary figure S4). To further confirm the role of CARS2 in the observed phenotype, we carried out retroviral transfection of normal *CARS2* into the cultured skin fibroblasts from the patient. The full-length, wild-type *CARS2* cDNA in a retroviral expression construct was introduced with high transfection efficiency (75%, see 'Methods'). The additional bands resulting from incomplete assembly of complex V in patient's cultured skin fibroblasts on BN-PAGE were no longer visible after normal *CARS2* cDNA transfection (figure 2C). This result indicated correction of the biochemical phenotype by the transfection with normal CARS2.

CARS2 mutation screening

Fifteen additional subjects with biochemical evidence of defects in mitochondrial transcription or translational were evaluated for *CARS2* mutations. Sanger sequencing of all 15 coding exons and exon–intron boundaries of *CARS2* did not identify potential pathogenic mutations. In order to potentially identify other subjects with a deficiency of CARS2, eight international mitochondrial and exome sequencing centres were contacted to evaluate

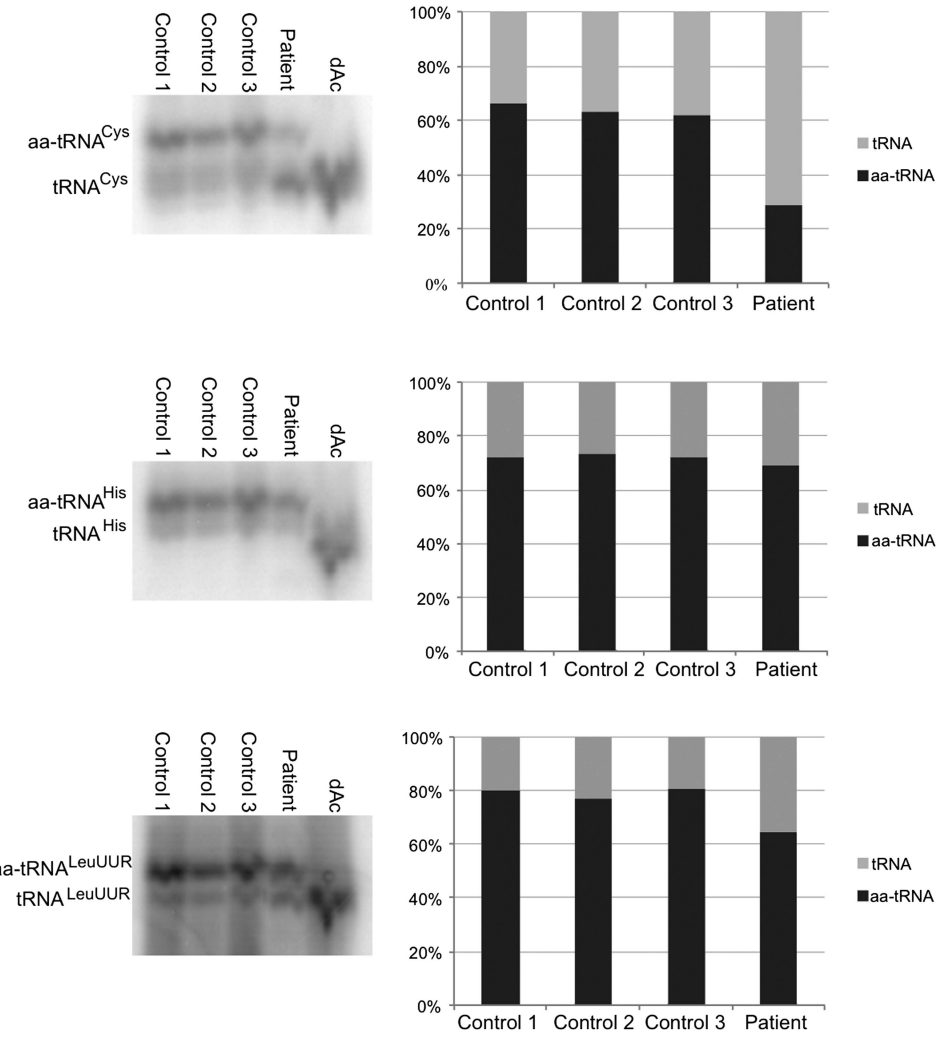

Figure 4 Mitochondrial tRNA aminoacylation analysis by northern blotting. Northern blot analysis of mitochondrial tRNA aminoacylation in total RNA samples from the patient and control fibroblasts (control 1–control 3). Membranes were hybridised with radioactive tRNA probes as indicated. 'dAc' indicates deacylated control sample. Densitometric quantification of the northern blots is shown on the right of each blot.

existing exome sequencing data. No other subjects with potential *CARS2* mutations were identified.

DISCUSSION

Disorders of mitochondrial translation are an increasingly recognised cause of combined respiratory chain enzyme deficiency.[25] Mitochondria possess their own translation machinery, which includes ribosomal and tRNAs encoded by mtDNA. In addition, mitochondrial translation requires nuclear encoded proteins including post-transcriptional tRNA modifiers, ARSs, mitochondrial ribosomal proteins, translation initiation factors, elongation factors and peptide release factors.[25] To date, defects in 36 nuclear-encoded mitochondrial translation genes have been implicated in human disease (table 1).

ARSs are key enzymes in the translation of mRNA into proteins since they catalyse the specific attachment of each of the 20 amino acids to the corresponding tRNA. ARSs are ubiquitously expressed enzymes that are present in most species ranging from bacteria to humans. Because protein translation also occurs in the mitochondria, ARSs activity is required in these organelles.[31] To facilitate their import, nuclear-encoded ARSs carry a mitochondrial targeting sequence within the mitochondrial ARSs (mt-ARSs) polypeptide. In total, 17 of the 37 nuclear-encoded ARSs genes including *CARS2* are exclusively mitochondrial targeted, two are bi-functional in cytoplasm and mitochondria, whereas one of the mt-tRNA (Gln) is aminoacylated via an indirect pathway.[32] Thus, the mutations in *CARS2* detected in our patient, both of which are predicted to be deleterious, are likely to lead to defects in mitochondrial translation.

Leukodystrophy is a common finding in mt-ARSs deficiency, but the clinical spectrum also includes cardiomyopathy (*AARS2*), myopathy-lactic acidosis-sideroblastic anaemia (*YARS2*) and pontocerebellar hypoplasia (*RARS2*).[33 34] The clinical phenotype in our patient was consistent with a severe mitochondrial epileptic encephalopathy with a complex movement disorder including severe oculogyric dystonia and mycolonus. Mild liver dysfunction with low albumin and steatosis has been noted in patients with *POLG* mutations. Defects in another mt-ARSs, *FARS2*, have also been associated with a very similar phenotype, which included fatal mitochondrial infantile-onset Alpers-like encephalopathy,[35 36] and epileptic encephalopathy was also noted in a patient with mutations in *VARS2*.[37] Recently, two siblings with severe myoclonic epilepsy and progressive cognitive decline were described with homozygous mutations in *CARS2*.[38] These siblings presented at 9 and 5 years, respectively, and were reported to make academic progress prior to onset of symptoms. Our patient presented in the neonatal period, and the clinical course was marked by a complex movement disorder not described in the previous report. All of the patients thus far described have a complex and progressive epileptic disorder with global brain atrophy noted on MRI. The similarity between the phenotypes of the two siblings and our patient lends further support to the causality of the *CARS2* mutations detected in our patient.

Table 1 Nuclear-encoded genes associated with defects in mitochondrial translation

Gene	OMIM	Function	Clinical symptoms
AARS2	612035	Aminoacyl-tRNA synthetase	Cardiomyopathy
CARS2	612800	Aminoacyl-tRNA synthetase	Mitochondrial encephalopathy
DARS2	610956	Aminoacyl-tRNA synthetase	Leukoencephalopathy
EARS2	612799	Aminoacyl-tRNA synthetase	Leukoencephalopathy
FARS2	611592	Aminoacyl-tRNA synthetase	Mitochondrial encephalopathy
HARS2	600783	Aminoacyl-tRNA synthetase	Ovarian dysgenesis, sensorineural hearing loss
IARS2	612801	Aminoacyl-tRNA synthetase	Cataracts, sensorineural hearing loss, encephaloneuromyopathy
LARS2	604544	Aminoacyl-tRNA synthetase	Ovarian dysgenesis, sensorineural hearing loss
MARS2	609728	Aminoacyl-tRNA synthetase	Ataxia
NARS2	612803	Aminoacyl-tRNA synthetase	Myopathy, encephalopathy
RARS2	611524	Aminoacyl-tRNA synthetase	Pontocerebellar hypoplasia
SARS2	612804	Aminoacyl-tRNA synthetase	Hyperuricemia, pulmonary hypertension, renal failure, alkalosis
TARS2	612805	Aminoacyl-tRNA synthetase	Mitochondrial encephalopathy
VARS2	612902	Aminoacyl-tRNA synthetase	Mitochondrial encephalopathy
YARS2	610957	Aminoacyl-tRNA synthetase	Myopathy, lactic acidosis, sideroblastic anaemia
GFM1	606639	Elongation factor	Hepatoencephalopathy
TSFM	604723	Elongation factor	Encephalopathy, cardiomyopathy
TUFM	602389	Elongation factor	Mitochondrial encephalopathy
MTFMT	611766	Initiation and elongation factor	Leigh syndrome
MRPL3	607118	Mitochondrial ribosomal protein	Cardiomyopathy
MRPL44	611849	Mitochondrial ribosomal protein	Cardiomyopathy
MRPS16	609204	Mitochondrial ribosomal protein	Agenesis corpus callosum, lactic acidosis
MRPS22	605810	Mitochondrial ribosomal protein	Hypotonia, cardiomyopathy, tubulopathy
C12orf65	613541	Peptide release factor	Encephalomyopathy
MTPAP	613669	Polyadenylation of mRNA	Spastic ataxia
LRPPRC	607544	Post-transcriptional regulation	Leigh syndrome
GTPBP3	608536	Post-transcriptional tRNA modification	Cardiomyopathy, mitochondrial encephalopathy
MTO1	614667	Post-transcriptional tRNA modification	Cardiomyopathy
PUS1	608109	Post-transcriptional tRNA modification	Myopathy, lactic acidosis, sideroblastic anaemia
TRIT1		Post-transcriptional tRNA modification	Mitochondrial encephalopathy
TRMU	610230	Post-transcriptional tRNA modification	Liver failure, deafness
ELAC2	605367	Post-transcriptional tRNA processing	Cardiomyopathy
HSD17B10	300256	Post-transcriptional tRNA processing	Cardiomyopathy, mitochondrial encephalopathy
PNPT1	610316	RNA turnover (or RNA import)	Encephalomyopathy, deafness
TACO1	612958	Translation activation	Leigh syndrome
RMND1	614917	Unknown	Encephaloneuromyopathy

In several mt-ARSs defects, a very limited phenotype has been observed in the skin fibroblasts from patients, including normal respiratory chain enzyme activities, normal BN-PAGE gel analysis and normal mitochondrial translation assays.[37 39 40] However, the identification of a functional defect using BN-PAGE in the proband's cultured skin fibroblasts provided us a means to evaluate the causality of a novel gene through correction of the functional defect by transfection with the native gene and mitochondrial tRNA aminoacylation analysis by northern blotting. Furthermore, a mouse strain carrying homozygous missense mutations in *Cars2* appears to have neurological phenotypes including tremors, induced hyperactivity and head bobbing.[41 42] These neurological phenotypes in the mouse appear to be age-related and correlate well with the complex movement disorders and regression observed in our patient. The analysis of such model organisms at the organismal, tissue and cellular levels will lead to a better understanding of the role of *CARS2* in the aetiology of the disease phenotype observed in our patient.

The identification of the genetic cause of severe mitochondrial infantile epileptic syndromes is difficult when mutations in *POLG* and *C10orf2* (*TWINKLE*) have been excluded. Often, the biochemical abnormalities are only present in liver despite minimal symptoms of liver dysfunction such as low albumin levels. Therefore, in patients with neurodegenerative disease, enzyme studies should include liver as well as skeletal muscle.[43 44] Defects in mitochondrial translation, particularly *FARS2*, *VARS2* and *CARS2*, expand the known causes of this devastating condition.

In summary, we have identified compound heterozygous mutations in *CARS2* in a patient with a combined respiratory chain enzyme deficiency using exome sequencing, followed by relevant functional studies to support the genetic finding. This report suggests that mutations in *CARS2* are an important cause of a mitochondrial epileptic syndrome and highlights the important contribution of high-throughput sequencing in the genetic diagnosis of suspected mitochondrial disorders.

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