A complex V ATP5A1 defect causes fatal neonatal mitochondrial encephalopathy

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Whole exome sequencing is a powerful tool to detect novel pathogenic mutations in patients with suspected mitochondrial disease. However, the interpretation of novel genetic variants is not always straightforward. Here, we present two siblings with a severe neonatal encephalopathy caused by complex V deficiency. The aim of this study was to uncover the underlying genetic defect using the combination of enzymatic testing and whole exome sequence analysis, and to provide evidence for causality by functional follow-up. Measurement of the oxygen consumption rate and enzyme analysis in fibroblasts were performed. Immunoblotting techniques were applied to study complex V assembly. The coding regions of the genome were analysed. Three-dimensional modelling was applied. Exome sequencing of the two siblings with complex V deficiency revealed a heterozygous mutation in the ATP5A1 gene, coding for complex V subunit α. The father carried the variant heterozygously. At the messenger RNA level, only the mutated allele was expressed in the patients, whereas the father expressed both the wild-type and the mutant allele. Gene expression data indicate that the maternal allele is not expressed, which is supported by the observation that the ATP5A1 expression levels in the patients and their mother are reduced to ~50%. Complementation with wild-type ATP5A1 restored complex V in the patient fibroblasts, confirming pathogenicity of the defect. At the protein level, the mutation results in a disturbed interaction of the α-subunit with the β-subunit of complex V, which interferes with the stability of the complex. This study demonstrates the important value of functional studies in the diagnostic work-up of mitochondrial patients, in order to guide genetic variant prioritization, and to validate gene defects.

Keywords: mitochondrial disease; complex V; next generation sequencing; functional genomics

Abbreviation: CCCP = carbonyl cyanide 3-chlorophenyl hydrazone

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Introduction

Complex V (also known as mitochondrial ATP synthase, EC 3.6.3.14) is a multi-subunit enzyme complex consisting of two functional domains, $F_0$ and $F_1$, connected by two stalks. The membrane-embedded $F_0$ domain functions as a proton channel and consists of 10 subunits, $a$ (subunit 6), $b$, $c$, $d$, $e$, $f$, $g$, OSCP, A6L (subunit 8), and F6. The catalytic domain $F_1$ is composed of subunits $\alpha$, $\beta$, $\gamma$, $\delta$, $\epsilon$, and a loosely attached inhibitor protein IF1. The $F_1$ domain uses the electrochemical gradient generated by the mitochondrial respiratory chain to convert ADP into ATP, the final step of the oxidative phosphorylation (Collinson et al., 1996; Houstek et al., 2009). To date, complex V deficiencies have been described in the mitochondrial DNA encoded ATP6 (MT-ATP6) (MIM +516060) and ATP8 (MT-ATP8) (MIM +516070) genes (www.mitomap.org). ATP synthase deficiency due to the nuclear encoded ATP12 (MIM 604273) and TMEM70 (MIM 612418) genes has also been reported (De Meirleir et al., 2004; Cizkova et al., 2008). Interestingly, only one mutation has hitherto been found in a nuclear encoded structural complex V subunit (Mayr et al., 2010), ATP5E (MIM 606153). Most complex V genes, however, are not routinely screened in a diagnostic setting since complex V deficiency is not a common oxidative phosphorylation (OXPHOS) deficiency and there is a large number of candidate genes (Rodenburg, 2011).

Recent advances in next-generation sequencing can counter the latter issue. Since most Mendelian disorders are caused by exonic mutations or splice-site mutations, whole exome sequencing can be applied to detect genetic defects underlying monogenic inherited disorders (Gilissen et al., 2011; Majewski et al., 2011). On the one hand, whole exome sequencing can be applied in an unbiased manner, based only on a clinical phenotype. The interpretation of tens of thousands of genomic variants, however, is still challenging. Bioinformatics analysis tools and data filtering criteria are used to identify causative mutations. For instance, when a recessive mode of inheritance is suspected, only the genes carrying homozygous or compound heterozygous variants are selected (Gotz et al., 2011; Gilissen et al., 2012). On the other hand, in the case of mitochondrial disorders, biochemical data of the OXPHOS system can be used to guide whole exome sequencing analysis. The latter approach has been applied in this study, describing two siblings with severe neonatal encephalopathy and an isolated complex V deficiency. Whole exome sequencing was performed to uncover the genetic defect, which was subsequently validated by functional complementation analysis. We show that mitochondrial biochemistry preceding whole exome sequencing analysis provides a useful guide for the analysis of whole exome sequencing data, and subsequently demonstrate the value of functional assays to validate novel genetic defects.

Patients and methods

Patients

We report two patients from a Dutch non-consanguineous family with the same clinical phenotype. Pregnancy and delivery were uneventful in both cases. They both died in the first weeks of life due to a severe encephalopathy characterized by intractable seizures (apnoeic spells). Clinical examination showed no dysmorphic features or organomegaly. Neurological examination at Day 1 revealed irritability, a high-pitched cry, a horizontal and vertical nystagmus, abnormal primitive reflexes, and tonus dysregulation. Brain MRI demonstrated a progressive and severe encephalopathy characterized by hyperdense thalami and subcortical densities in the eldest sibling. The EEG showed abnormal slow waves in the right hemisphere. Post-mortem anatomopathological examination revealed extensive cerebral damage: the cerebellum was small and the consistency of the occipital lobe was weakened. There was a cystic degeneration of the white matter, especially occipital and temporoparietal. The pons and brainstem were damaged. The lungs were hypoplastic, there were small renal cysts, and skeletal muscle contained small lipid droplets. The cerebral, renal and skeletal muscle lesions indicated a mitochondrial disease. The MRI of the younger patient was characterized by progressive frontal and parieto-occipital damage including the posterior limb of the internal capsule, the pyramidal tract and basal ganglia, mainly in the right hemisphere (Fig. 1). The cerebellum and pons were also damaged. Metabolic screening in blood, urine and CSF showed no abnormalities in both cases. The excretion in urine of 3-methylglutaconic acid was not elevated.

Cell cultures

Fibroblasts were cultured in medium 199 (Gibco®, Invitrogen Corporation) supplemented with 10% foetal calf serum and penicillin/streptomycin (100 U/ml and 100 µg/ml, respectively). 293FT cells (Invitrogen) were grown in Dulbecco’s modified Eagle’s medium containing 4.5 g/l glucose, 10% foetal calf serum, 4 mM L-glutamine, 1% penicillin/streptomycin, and 500 µg/ml geneticin (G418). During transductions, the medium did not contain penicillin/streptomycin and geneticin (following Invitrogen recommendations).

Biochemical assays

Mitochondrial respiration in digitonin-permeabilized cells was analysed in the presence of a substrate mix containing pyruvate and malate, using the fluorosence probe Mitox (Luxcell), 96-wells plates, and a fluorescence plate reader as described (Jonckheere et al., 2010). The complex V (or mitochondrial ATPase) activity was measured spectrophotometrically in mitochondria isolated from patient and control fibroblasts as described (Jonckheere et al., 2008). The activities of the mitochondrial respiratory chain enzymes and citrate synthase were measured spectrophotometrically in fibroblasts of the patients according to described protocols (Cooperstein and Lazarow, 1951; Sere, 1969; Mourmans et al., 1997; Janssen et al., 2007; Rodenburg, 2011). All measurements were performed in duplicate (coefficient of variation < 15%).

Complex V assembly and activity

One-dimensional 10% SDS-PAGE, 5–15% Blue Native PAGE and 2D 10% SDS-PAGE after Blue Native PAGE were performed as described previously (Schagger and von Jagow, 1991; Nijtmans et al., 2002). Lanes were loaded with 30 µg (SDS analysis) or 40 µg (Blue Native analysis) of solubilized mitochondrial protein (Smith et al., 1985). After electrophoresis, gels were processed further for immunoblotting as described by Nijtmans et al. (2002). For western blotting, antibodies used were raised against V5 (Invitrogen), mitochondrial complex V.
subunits alpha, beta, OSCP, and d; complex I subunit NDUFA9, complex IV subunit IV, complex III subunit core 2, and CoII-70 kDa (complex II) (MitoSciences). For the in-gel activity assay of ATP hydrolysis the gel was incubated overnight at room temperature with the following solution: 35 mM Tris, 270 mM glycine, 14 mM MgSO₄, 0.2% Pb(NO₃)₂, and 8 mM ATP, pH 7.8 (Nijtmans et al., 2002).

Molecular genetic analysis

Whole exome sequencing

We sequenced the exomes (Sure Select v2, 50 Mb, Agilent) of the two affected relatives, Patient 1 (DDNA10-0976) and Patient 2 (DDNA10-0926) on a SOLiDTM 4 system (Life Technologies). We sequenced a total of 126 million and 157 million reads of 50 bp, respectively. Sequence data were mapped to the hg19 reference genome with the SOLiDTM bioscope software version 1.3 (Supplementary Table 1A). This resulted in 80% and 83% of the targets being covered at least 10 times, and a median coverage of 55- and 71-fold, respectively (Supplementary Table 1A).

Variants were called by Bioscope v1.3 and annotated using a custom-made bioinformatics pipeline (Gilissen et al., 2010; Hoischen et al., 2011). Variants were prioritized based on predicted amino acid consequences and overlap with common variation (dbSNP v.130, in-house variant database consisting of ~1200 exomes from patients with various rare Mendelian disease and containing more than 1.2 million variants) (Supplementary Table 1B). All private non-synonymous and splice site variants that were present in both affected individuals are shown in Supplementary Table 1C.

ATP5A1 genomic and coding DNA analysis

Total DNA from blood and cultured fibroblasts was extracted using the salting out procedure for human DNA extraction (Miller et al., 1988). The coding region in the genomic DNA of the ATP5A1 gene was PCR amplified using 10 primer pairs (Supplementary Table 2A). PCR conditions were 95°C for 10 min (denaturation), followed by 95°C for 30 s, 60°C for 30 s and 72°C for 1 min for a total of 35 cycles. Sequence analysis of PCR amplified products was performed on an ABI3730 automatic capillary sequencer using BigDye® terminator chemistry (Applied Biosystems). A similar approach was applied for DNA sequence analysis of the ATP5A1 5’ UTR or promoter regions (preceding exons 1 and 2), the ATP5A1 3’ UTR region and the ATP5A1 intron regions (primer sequences are available upon request). RNA from blood and cultured fibroblasts was extracted as described (Miller et al., 1988). Reverse transcriptase PCR was performed to produce coding DNA followed by PCR amplification (Supplementary Table 2C). Sequence analysis was subsequently performed as described above. Real-time quantitative PCR analysis of coding DNA of ATP5A1 and the reference gene GAPDH was performed using a CFX96™ Real-time system/C1000™ Thermal Cycler and Sso Advanced™ SYBR® Green Supermix (Bio-Rad) following the manufacturer’s specifications. Primers were designed to span exon-exon boundaries (Supplementary Table 2C). Two dilutions of each sample were analysed in duplicate using the following PCR conditions: 95°C for 10 min (denaturation), followed by 95°C for 30 s, 60°C for 30 s and 72°C for 1 min for a total of 35 cycles. Subsequently, a melting curve was performed to check for the specificity of the PCR reactions.

Copy number variation detection by single nucleotide polymorphism micro array

Affymetrix CytoScan HD array was performed to search for genome-wide deletions or duplications with a median resolution of 20 kb (Human Genome Build hg19, UCSC genome browser, February 2009).

Figure 1

Brain MRI of Patient 2 at Day 11 (A) and Day 24 (B), axial T2 weighted images, showing extensive signal abnormalities in large parts of the right hemisphere, including white matter, posterior limb of the internal capsule (asterisk), basal ganglia and thalamus.
Multiplex ligation-dependent probe amplification analysis

To detect exon deletions or duplications in ATP5A1, multiplex ligation-dependent probe amplification analysis was performed as described (Schouten et al., 2002).

Cloning of human ATP5A1 gene and lentiviral complementation

The full length open reading frame for ATP5A1 was amplified by PCR from I.M.A.G.E. coding DNA clone IRATp970G0784D (Source BioScience) using specific primers with Gateway attB flanking sites without a stop codon. The product was cloned into pDONR201 using the Gateway BP Clonase II Enzyme Mix (Invitrogen). The resulting entry clone was verified by sequence analysis and subsequently recombined with the pLent6.2V5-DEST destination vector (Invitrogen) using the Gateway LR Clonase II Enzyme Mix (Invitrogen). The Gateway BP and LR reactions were performed according to the manufacturer’s instructions. The resulting pLent6.2-V5-ATP5A1 or the control construct pLenti6.2-V5-AcGFP were transduced into 10 cm dishes 293FT cells together with a packaging mix consisting of pLP1, pLP2 and pLP/VSVG using Lipofectamine 2000 (Invitrogen). Twenty-four hours after transduction the medium was refreshed, and 72 h after transduction supernatants containing the viral particles were harvested. Supernatants were cleared by centrifugation (5 min at 3000g) and the viral particles were stored at −80°C. Infections were performed on fibroblasts in 75 cm² flasks with 2 ml of virus containing supernatant in the presence of 6μg/ml polybrene (Sigma). Twenty-four hours after infection the medium was refreshed, and 48 h after transduction the selection medium was added [M199, 20 % foetal calf serum, penicillin/streptomycin (100 U/ml and 100μg/ml, respectively), 2μg/ml blasticidin (InvivoGen)]. Cells were selected for 14 days, during which the mock infected cells (without virus) died. Blasticidin resistant cells were used for biochemical analysis within six passages after transduction.

Three-dimensional modelling

A 3D model of the human F₁-ATPase, including the Arg329Cys mutation, was created using the automatic homology modelling script in the YASARA & WHAT IF Twinset (Vriend, 1990; Krieger et al., 2002). As a template for this model we used PDB structure 1BMF (Abrahams et al., 1994), containing the structure of the bovine F₁-ATPase, with an α-subunit that is 98% identical to its human orthologue.

Results

Biochemical assays

A decreased oxygen consumption rate was observed in cultured fibroblasts of both patients (Fig. 2A and B) compared with control cells (Fig. 2C). A substantial increase was seen after addition of an uncoupler (2μM carbonyl cyanide 3-chlorophenyl hydrzone, CCCP) in the fibroblasts of both patients (Fig. 2A and B). This is not observed in control cells (Fig. 2C) and indicates a complex V deficiency (Jonckheere et al., 2010).

Measurements of the mitochondrial respiratory chain enzyme activities were performed in cultured fibroblasts (no muscle tissue was available for both patients) and displayed an isolated complex V deficiency in both cases (Table 1).
Complex V assembly and activity

We performed blue native PAGE and 2D SDS-PAGE after blue native PAGE on fibroblasts of the two patients and a healthy control subject. Blue native PAGE showed that the amount of complex V was strongly reduced in the patients compared with the control (Fig. 3A). The in-gel activity assay of ATP hydrolysis demonstrated a reduced complex V activity (Fig. 3A). Two-dimensional SDS-PAGE after blue native PAGE showed holocomplex V, with no accumulation of subcomplexes (Fig. 3B). One-dimensional SDS-PAGE showed that the levels of individual complex V subunits were decreased (Fig. 3C), which is in accordance with the previously described proteolytic removal of unassembled F1 subunits in HeLa cells depleted in the β-subunit of complex V (Rak et al., 2011). In conclusion, these data indicate a disturbed complex V assembly in an early stage (at the F1 level).

Molecular genetic analysis

Genetic analysis did not reveal mutations in the mitochondrial DNA or nuclear genes previously known to cause complex V deficiency (ATP12, TMEM70, ATP5E). Therefore, whole exome sequencing was performed, revealing 22 private variants shared by both affected siblings, most of which appeared to be heterozygous, with the exception of a variant in ITPR3 that showed apparent homozygosity in both patients. This variant was considered to be an unlikely candidate to explain the clinical features and complex V deficiency in the two patients, as the gene encodes the inositol 1,4,5-trisphosphate receptor, type 3, which is a modulator of hepatic glucose production and has been associated with both type 1 diabetes and the auto-immune disorder systemic lupus erythematosus. Of the other 21 private variants, only three encode a protein known to be localized in mitochondria (ATP5A1, UCP3 and ACLY). Only the ATP5A1 variant is compatible with the complex V deficiency present in the fibroblasts of both patients. Although we cannot exclude a role of a mutation in one of the other 21 genes in complex V deficiency, we did not find any evidence in the experimental literature for this. Also STRING database analysis (Franceschini et al., 2013) did not reveal evidence for an interaction with complex V. At the lowest stringency (0.15), none of the gene order conservation, gene fusion, co-expression, co-evolution, and physical interaction data from the STRING database contains predictions for interactions between any of the proteins in the list and any of the complex V proteins or their known assembly factors. The mutation at c.985C>T (p.Arg329Cys) (Ref Seq accession number NM_001001937) in exon 9 of ATP5A1 (chromosome 18q21, MIM *164360) has a high PhyloP score of 5.7 (Pollard et al., 2010) and a Grantham score of 180 (Grantham, 1974), indicative of evolutionary conservation of the wild-type Arg329 residue and physicochemical dissimilarities between the mutant Cys329 and wild-type Arg329 residues, respectively. The mutation is not present in any of the 1200 other exomes currently in the Nijmegen database. The heterozygous mutation was confirmed by Sanger sequencing. The father was also heterozygous for this mutation, whereas the mother did not carry this mutation (Fig. 4). Next, coding DNA analysis revealed that only the mutated allele was expressed in both patients, whereas the father expressed both the mutated and the wild-type allele and mother expressed only the wild-type coding sequence (Fig. 4). This indicates that the allele inherited from the mother is not expressed in both patients. There were no heterozygous single nucleotide polymorphisms present in the exonic and untranslated regions of the mother’s ATP5A1 coding DNA that could be used to follow the expression of the maternal alleles in the two patients and in the mother. PCR experiments using different combinations of primers did not provide evidence for a splicing defect of the maternal allele (data not shown). Quantitative PCR analysis showed that the expression levels of ATP5A1 messenger RNA in fibroblasts of the two patients are half of that in control fibroblasts (Fig. 5). In addition, the expression level of ATP5A1 messenger RNA in blood cells of the mother was ~60% compared with the father. These data are compatible with a gene expression defect of the maternal ATP5A1 allele inherited by both patients. The loss of the maternal ATP5A1 transcript in the two patients was not due to mutations in the open reading frame of ATP5A1 (all exons were checked by Sanger sequencing), or due to mutations in the 5’UTR [promoter regions sequenced from g.43677380-43679525 (preceding exon 2) and from g.43684103-43684199 (preceding exon 1) (both regions Chr18, GRCh37/hg19 = NCBI Build 37)] and 3’UTR regions (data not shown). The karyotype was normal (data not shown). A CytoScan HD array analysis showed no pathogenic alterations (data not shown). Multiplex ligation-dependent probe amplification analysis did not show deletions or duplications in ATP5A1 alleles (data not shown). Sequencing of branch points, of highly conserved introns and of non-coding regions potentially harbouring transcription binding sites in ATP5A1 did not reveal mutations, nor did it reveal single nucleotide polymorphisms that were present only in the mother but not in the father (data not shown).

Table 1 Enzymatic analysis of the activities of complex V (mitochondrial ATPase), the respiratory chain enzymes and citrate synthase in fibroblasts of Patients 1 and 2

<table>
<thead>
<tr>
<th>Component</th>
<th>Patient 1 (mU/U complex IV)</th>
<th>Patient 2 (mU/U complex IV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complex V</td>
<td>116 (209–935)</td>
<td>76 (209–935)</td>
</tr>
<tr>
<td>Complex I</td>
<td>140 (110–260)</td>
<td>126 (110–260)</td>
</tr>
<tr>
<td>Complex II</td>
<td>661 (536–1027)</td>
<td>698 (536–1027)</td>
</tr>
<tr>
<td>Complex III</td>
<td>2311 (1270–2620)</td>
<td>2431 (1270–2620)</td>
</tr>
<tr>
<td>Complex IV</td>
<td>929 (680–1190)</td>
<td>918 (680–1190)</td>
</tr>
<tr>
<td>Citrate synthase</td>
<td>171 (144–257)</td>
<td>179 (144–257)</td>
</tr>
</tbody>
</table>

Reference ranges in brackets.
except for a heterozygous variant c.-49 + 418C > T (GRCh37: g.43683685G > A) in the first intron of the gene, which was found in both patients and in the mother. It was located in an Alu-repeat sequence of the Alu-Sx subfamily (identity to the Alu-Sx subfamily is 92%). There is no indication of this variant affecting gene regulation. It is positioned at a distance of 5.4 kb from the core promoter of the ATP5A1 gene and is not required for the basal activity of this promoter (Vander Zee et al., 1994). In the Encyclopedia of DNA Elements (ENCODE), no transcription factor binding sites are present in this region of the gene, nor is this region accessible to DNase I (Dunham et al., 2012). Furthermore, based on sequence conservation data, this region of the gene does not contain any predicted transcription factor binding sites (Lindblad-Toh et al., 2011). Importantly, the presence of this sequence variant in both children and in the mother shows that both children inherited the same allele from the mother.

**Complementation of complex V deficiency by wild-type ATP5A1**

To confirm that the lack of wild-type ATP5A1 expression is responsible for the complex V deficiency in the patients, we...
transduced fibroblasts of the index patients and a healthy control subject using a lentiviral system. Complementation of patient fibroblasts with wild-type ATP5A1 completely normalized complex V amount (Fig. 6) and activity (Table 2).

Modelling of p.Arg329Cys in ATP5A1

The pathogenicity of the p.Arg329Cys in ATP5A1 was corroborated by protein modelling. The Arg329 residue is predicted to form a hydrogen bond with a carbonyl group of the Gly323 residue of the β-subunit (Fig. 7). As the F₁-ATPase has three α subunits and three β subunits, the p.Arg329Cys mutation would abolish three stabilizing interactions in the complex. This predicted loss of stability of the complex is in line with the western blot results showing degradation of unassembled complex V subunits. Furthermore, the Arg329 residue of the α-subunit is very well conserved, even among bacterial orthologues of ATP5A1 (data not shown), underlining its importance for the functioning of the complex.

Discussion

Clinical phenotype

The severe neonatal encephalopathy of the patients described here resembles the clinical picture of the patient with a mutation in the complex V assembly gene ATP12 (De Meirleir et al., 2004). The latter patient however also presented with dysmorphic features, an enlarged liver and hypoplastic kidneys, features that were not present here. It should be noted that post-mortem anatomopathological examination of the eldest sibling revealed renal cysts and hypoplastic lungs. Remarkably, there is a predominance of the cerebral lesions in the right hemisphere in the patients described here. Hypertrophic cardiomyopathy, which is often seen in...
complex V deficiency (Cizkova et al., 2008; Jonckheere et al., 2008), was not documented.

Pathogenicity

The ATP5A1 defect found in both patients comprises a missense mutation in the paternal allele and an undiscovered mutation leading to non-expression of the maternal allele. Pathogenicity of the sequence variant in an Alu-repeat in intron 1 of the maternal allele does not seem likely. Instead, a mutation could be situated in hitherto unknown enhancers or in silencers who are situated upstream or downstream of the ATP5A1 gene. Alternative splicing of the ATP5A1 gene could possibly play a role, although we found no evidence for this. Several lines of evidence point towards

Table 2 Complementation with wild-type ATP5A1 restores complex V activity in patient fibroblasts

| Complex V activity (mU/U citrate synthase, reference range 193–819) |
|--------------------------|--------------------------|
| Patient 1 – GFP         | 118                      |
| Patient 1 – ATP5A1      | 337                      |
| Patient 2 – GFP         | 118                      |
| Patient 2 – ATP5A1      | 381                      |
| Control – GFP           | 748                      |
| Control – ATP5A1        | 665                      |

\*Patient 1 – GFP: negative control construct pLent6.2-V5-AcGFP.
Reduced activities indicated in bold.
pathogenicity of the lack of normal ATP5A1 expression. Clinically, there is a severe and fatal neonatal encephalopathy, while biochemically, an isolated complex V deficiency was found. At the messenger RNA level, only the mutated allele was expressed in both patients. Modelling of the mutation showed a disturbed interaction between the \( \alpha \) and \( \beta \) subunits of complex V. This is predicted to lead to instability of the complex, which is in line with the western blot results showing a severely decreased holocomplex V and reduced subunit protein levels. The mutation is situated in a conserved region of the protein. Finally, complementation of the ATP5A1 defect restored the amount and activity of complex V. Interestingly, in a mouse model it has been shown that a defect in ATP5A1 causes a lethal phenotype as well as a 4 bp duplication in exon 3 of ATP5A1 leading to a stop codon in exon 4 and therefore an unstable transcript, resulted in death in utero (Baran et al., 2007).

Value of functional assays before and after whole exome sequencing

This study demonstrates the added value of biochemical assays in the prioritization of genetic variants identified by exome sequencing, as part of the diagnostic work-up of suspected mitochondrial patients. The heterozygous mutation in ATP5A1, although it was not only found in the two patients but also in their father, was given the highest priority for further studies because of the known complex V deficiency in the patients. In general, we advocate to perform biochemical diagnostic investigations before commencing with whole exome sequencing in suspected mitochondrial patients, as the biochemical data can be very valuable in the prioritization of genetic variants, in combination with other features of genetic variants (Gotz et al., 2011; Gilissen et al., 2012). After whole exome sequencing, defining the true impact of new genomic variants by functional follow-up (e.g. coding DNA analysis, complementation studies) is mandatory. In our study, complementation of patient fibroblasts with wild-type ATP5A1 restored complex V activity, demonstrating that the ATP5A1 defect found in these patients is disease causing, even though the exact nature of one of the two ATP5A1 mutations remains unknown. This emphasizes the importance to mitochondrial medicine of functional studies to characterize novel genetic variants. This was also stated by Tucker et al. (2012), who showed by functional testing that a branch-site mutation in NUBPL was the main cause for lack of NUBPL expression and complex I deficiency, rather than a homozygous missense mutation identified by exome sequencing in the same gene of their patient. Also in other studies, the importance of functional validation of genetic variants identified by whole exome sequencing has been demonstrated (Calvo et al., 2010; Haack et al., 2012).

Taken together, we currently recommend functional studies before and after whole exome sequencing analysis to identify and confirm the pathogenicity of a mutation in patients with a mitochondrial disorder.

Implications

In summary, we report the first defect in ATP5A1, which encodes complex V subunit \( \alpha \), causing an isolated complex V deficiency with a distinct clinical phenotype, i.e. a severe neonatal encephalopathy leading to early death. Moreover, this study demonstrated that whole exome sequencing analysis in combination with functional assays preceding (enzyme activity analysis) and following
whole exome sequencing (coding DNA analysis, complementation studies) provide a powerful approach to identify pathogenic genetic defects in suspected mitochondrial patients.

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Supplementary material
Supplementary material is available at Brain online.

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