ORIGINAL ARTICLE

Mutation screening of 75 candidate genes in 152 complex I deficiency cases identifies pathogenic variants in 16 genes including *NDUFB9*

Tobias B Haack,[1,2] Florence Madignier,[2] Martina Herzer,[2] Eleonora Lamantea,[3] Katharina Danhauser,[2] Federica Invernizzi,[3] Johannes Koch,[4] Martin Freitag,[2] Rene Drost,[2] Ingo Hillier,[2] Birgit Haberberger,[2] Johannes A Mayr,[4] Uwe Ahting,[5] Valeria Tiranti,[3] Agnes Rötig,[6] Arcangela Iuso,[1] Rita Horvath,[7] Marketa Tesarova,[8] Ivo Baric,[9] Graziella Uziel,[10] Boris Rolinski,[5] Wolfgang Sperl,[4] Thomas Meitinger,[1,2] Massimo Zeviani,[3] Peter Freisinger,[11] Holger Prokisch[1,2]

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For numbered affiliations see end of article.

Correspondence to
Dr Holger Prokisch, Institute of Human Genetics, Technische Universität München, Trogerstrasse 32, 81675 Munich, Germany; prokisch@helmholtz-muenchen.de

TBH, FM, MH and EL contributed equally to this work.

Received 19 October 2011
Accepted 25 November 2011
Published Online First
26 December 2011

ABSTRACT

Background Mitochondrial complex I deficiency is the most common cause of mitochondrial disease in childhood. Identification of the molecular basis is difficult given the clinical and genetic heterogeneity. Most patients lack a molecular definition in routine diagnostics.

Methods A large-scale mutation screen of 75 candidate genes in 152 patients with complex I deficiency was performed by high-resolution melting curve analysis and Sanger sequencing. The causal role of a new disease allele was confirmed by functional complementation assays. The clinical phenotype of patients carrying mutations was documented using a standardised questionnaire.

Results Causative mutations were detected in 16 genes, 15 of which had previously been associated with complex I deficiency: three mitochondrial DNA genes encoding complex I subunits, two mitochondrial tRNA genes and nuclear DNA genes encoding six complex I subunits and four assembly factors. For the first time, a causal mutation is described in *NDUFB9*, coding for a complex I subunit, resulting in reduction in NDUFB9 protein and both amount and activity of complex I. These features were rescued by expression of wild-type *NDUFB9* in patient-derived fibroblasts.

Conclusion Mutant *NDUFB9* is a new cause of complex I deficiency. A molecular diagnosis related to complex I deficiency was established in 18% of patients. However, most patients are likely to carry mutations in genes so far not associated with complex I function. The authors conclude that the high degree of genetic heterogeneity in complex I disorders warrants the implementation of unbiased genome-wide strategies for the complete molecular dissection of mitochondrial complex I deficiency.

INTRODUCTION

Mitochondrial respiratory chain complex I is an NAD—ubiquinone oxidoreductase. Electron transfer from NAD to ubiquinone is coupled with proton translocation across the inner mitochondrial membrane,[1] thus contributing to construction of the electrochemical gradient essential for ATP synthesis, protein translocation and ion transport. In humans, complex I is composed of as many as 45 different protein subunits, a non-covalently bound flavin mononucleotide moiety, and eight iron—sulphur clusters, for a molecular mass approaching 1 MDa, by far the largest complex of the OXPHOS system.[2] Seven subunits are encoded by mitochondrial DNA (mtDNA) and 38 by nuclear DNA genes. A number of ancillary proteins—some of which are known, others only postulated—are essential for maturation, assembly and stability of complex I. Atomic-force microscopy and x-ray crystallographic studies have established the quaternary structure of complex I as an L-shaped object, consisting of two arms: a hydrophobic arm, which contains the seven mtDNA-encoded subunits, embedded in the inner mitochondrial membrane; and a hydrophilic, peripheral arm, protruding into the matrix and hosting the iron—sulphur clusters and the flavin mononucleotide moiety.[3]

Human respiratory chain diseases have an estimated incidence of 1 in 5000 live births, with impaired complex I enzyme activity being the most commonly observed biochemical defect (MIM 252010).[4] The clinical phenotype usually manifests in infancy or early adulthood and includes leukodystrophy, epilepsy, skeletal myopathy, cardiomyopathy, lactic acidosis and Leigh syndrome.[5 6]

To date, pathogenic mutations have been reported in all of the 14 evolutionarily conserved subunits of the catalytic core,[7] encoded by the seven mitochondrial genes—*ND1* (MIM 516000),[8] *ND2* (MIM 516001),[9] *ND3* (MIM 516002),[10] *ND4* (MIM 516003),[11] *ND4L* (MIM 516004),[12 13] *ND5* (MIM 516005)[14] and *ND6* (MIM 516006)[15]—and seven nuclear genes—*NDUFV1* (MIM 161015),[16] *NDUFV2* (MIM 600532),[17] *NDUFS1* (MIM 157655),[18] *NDUFS2* (MIM 602985),[19] *NDUFS3* (MIM 603846),[20] *NDUFS7* (MIM 601825)[21] and *NDUFS8* (MIM 602141).[22] Further causative mutations have been described in several nuclear DNA-encoded accessory subunits—NDUFS4 (MIM 602694),[23] NDUFS6 (MIM 603848),[24] NDUFA1 (MIM 300078),[25] NDUFA2 (MIM 602137),[26]

NDUFA10 (MIM 603835),[27] NDUFA11 (MIM 612638)[28] and NDUFA12[29]—and in nine nuclear-encoded ancillary proteins that are necessary for complex I assembly, maturation or stability—NDUFAF1 (MIM 606934),[30] NDUFAF2 (MIM 609653),[31] NDUFAF3 (MIM 612911),[32] NDUFAF4 (MIM 611776),[33] C8orf38 (MIM 612392),[34] C20orf7 (MIM 612360),[35] ACAD9 (MIM 611103),[36] NUBPL (MIM 613621) and FOXRED1 (MIM 613622).[37] Nuclear DNA-encoded defects are all inherited as autosomal recessive traits, with the exception of mutations in *NDUFA1*, an X-linked gene.[25]

The broad phenotypic spectrum, the sheer number of genes involved, and the still partial dissection of the disease genes underpinning the biochemical defect pose a major hurdle in the diagnostic workup. Most complex I-deficient patients lack a molecular definition in routine diagnostics.[38] In this study we have applied a high-throughput mutation screen of 75 known or putative disease genes in a cohort of 152 index patients with a biochemical and clinical phenotype consistent with complex I deficiency. The results include the confirmation of known mutations, the identification of new mutations, and the functional validation of a pathogenic mutation in a novel disease gene, *NDUFB9*, by complementation assays in cell cultures.

SUBJECTS AND METHODS

Subjects

Patients have been recruited in five European centres. All 152 individuals who entered the mutation screen had a clinical presentation supportive of mitochondrial disease.[38] Written informed consent was obtained from all participants or their guardians at the recruiting centre.

Respiratory chain activities were normalised to that of citrate synthase, an index of mitochondrial mass. All centres provided a mean value for corrected complex I activity derived from at least 10 healthy controls. Complex I deficiency was defined as residual complex I activity less than 2 SDs below the mean of controls, in homogenates of muscle biopsy samples, fibroblast cell cultures, or both.

Previously performed molecular routine diagnostics in the patients ranged from selective analysis of mitochondrial- and nuclear-encoded disease genes to whole mitochondrial genome sequencing in 20 cases. Only patients without a previous molecular diagnosis were included in this study. The results for seven patients have been published during the study. They harboured mutations in the *NDUFS1* (n=2),[39] *NDUFA1*,[40] *NDUFAF2*[41] and *ACAD9* (n=3)[42] genes.

Molecular analysis

High resolution melting curve analysis (HRMA) was performed on a LightScanner instrument (Idaho Technology, Salt Lake City, Utah, USA) as described previously.[43] PCR primers were designed using the ExonPrimer and the LightScanner Primer Design software package (Idaho Technology). We analysed more than 100,000 melting curves from 266 different amplicons in 152 DNA samples in duplicate or quadruplicate, covering exons encoding the 45 subunits of complex I, the mtDNA-encoded tRNAs and eight accessory factors including *NDUFAF1*, *NDUFAF2*, *NDUFAF4*, *NDUFAB1* (MIM 603836), *ECSIT* (MIM 608388), *ACAD9*, *FOXRED1* and *NUBPL*. Primer sequences and PCR protocols are available on request. Double-strand DNA binding LCGreen Plus dye (Idaho Technology) was added to the mastermix before PCR. Before the start of HRMA, a layer of 8 µl mineral oil was placed above the PCR products. From each group of DNA samples with altered melting curves compared with the average of multiple wild-types, at least three samples were sequenced and, in the case of detection of a potential mutation, samples with the same melting curve were sequenced with a BigDye Cycle sequencing kit (Applied Biosystems, Carlsbad, California, USA). Ninety of 152 DNA samples were run in four replicates—two with the standard protocol and two with the addition of control DNA—in order to increase sensitivity for homozygous mutations. A total of 286 amplicons were designed for the screen, but in 20 amplicons (7%) the specificity of the PCR or the melting curve profile was unsuitable for HRMA, and these were not further analysed. In <1%, the PCR failed and was repeated separately and analysed by Sanger sequencing. Identified mutations were verified in an independent experiment in the stock DNA.

For quality control, we added seven DNA samples from patients harbouring compound heterozygous mutations in one of the genes investigated in the analysis. Twelve of 14 (86%) disease alleles were correctly identified.

Complementation of new mutations

For the patients harbouring mutations in the *NDUFB9* gene, feline immunodeficiency virus-based lentiviral transduction with the full-length cDNA of *NDUFB9* was performed as described previously.[39]

Complex I, complex IV and citrate synthase activities were determined spectrophotometrically as described,[44] using a JASCO V-550 spectrophotometer. Three independent experiments were performed for each condition.

Immunoblot analysis was performed on mitochondria-enriched[45] cell lysates using the primary antibodies, anti-NDUFS1 (Santa Cruz Biotechnology, Santa Cruz, California, USA; sc-50132), anti-NDUFS3 (Mitosciences; MS-110), anti-NDUFA9 (Mitoscience, Eugene, Oregon, USA; MS-111), anti-NDUFB9 (Santa Cruz Biotechnology; sc-98030), anti-complex III subunit core 2 (Mitosciences; MS-304) and anti-porin (Mitosciences; MS-304). Secondary antibodies were anti-goat, anti-mouse and anti-rabbit (Amersham Bioscience, Little Chalfont, UK). The signal was detected using ECL (Amersham Bioscience).

RESULTS

Genetic diagnoses in known complex I disease genes

Genetic screening of the 75 genes revealed causative mutations in 18% of the patients (27/152), affecting 15 genes, which have previously been associated with complex I deficiency: three mtDNA genes encoding complex I subunits, two mt-tRNA genes, six nuclear genes encoding complex I subunits and four genes encoding assembly factors (figure 1).

A total of 10 previously reported mtDNA missense mutations were detected in the *ND3* (n=4), *ND5*, *ND6* (n=2), *MTTL1* (n=2) and *MTTC* genes. All mutations met the pathogenicity criteria specified by Mitchell *et al*.[13] The supporting evidence for the pathogenicity of the mutations and the percentage of heteroplasmy estimated from Sanger sequencing are provided in table 1.

All mothers of the patients showed the mutation, with an estimated heteroplasmy <50% in blood. The results of another 13 patients harbouring rare variants of unknown significance not annotated in MITOMAP are listed in online supplementary table 1.

Homozygous and compound heterozygous variants (referred to as 'recessive-type' mutations below) were detected in 17 index patients in known nuclear disease genes, namely *ACAD9* (3×), *FOXRED1*, *NDUFA1*, *NDUFA10*, *NDUFAF2*, *NDUFAF4*, *NDUFS1* (6×), *NDUFS2*, *NDUFS4* and *NDUFS6*. The identified mutations were absent from pilot data from the 1000 genomes project, dbSNP[54] (v130), and at least 200 control chromosomes,

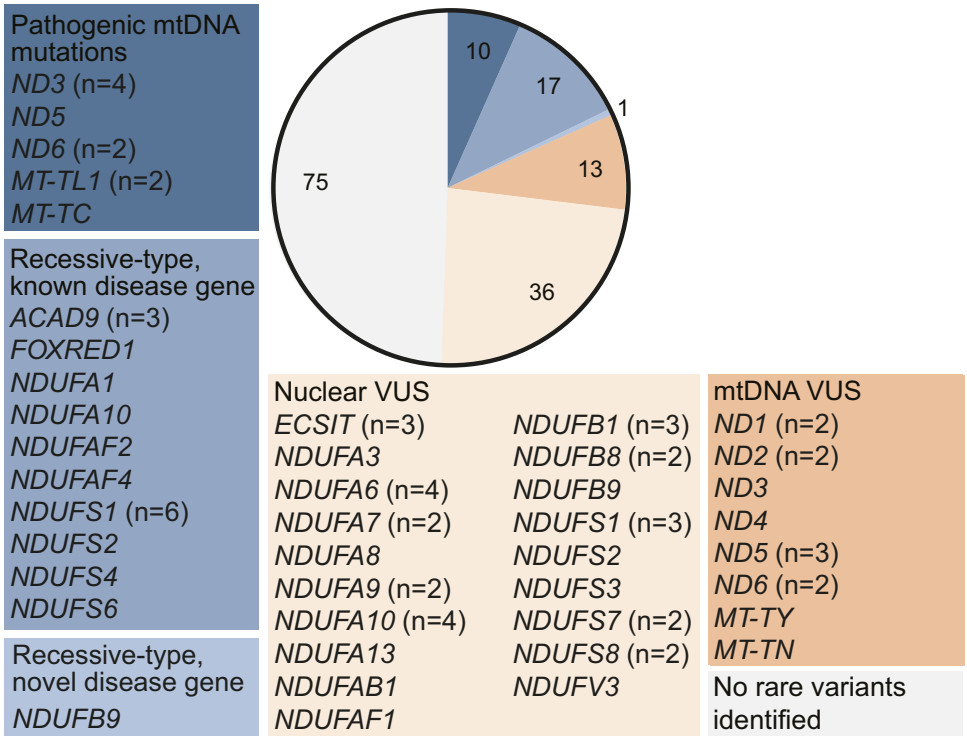

Figure 1 Mutational spectrum of complex I deficiency in 152 index patients. These patients lack a previous genetic diagnosis are classified by presumed pathogenic variants identified per gene: (i) pathogenic mtDNA mutations, recessive-type mutations in (ii) known nuclear encoded disease genes and (iii) a previously undescribed disease gene, variants of unknown significance (VUS) in (iv) mtDNA and (v) nuclear DNA, and (vi) subjects with no rare variants identified. Boxes list genes harbouring likely damaging variants, with the number of affected individuals in parentheses. Recessive-type mutation refers to homozygous and compound heterozygous mutations consistent with an autosomal recessive mode of inheritance.

except for two (rs1801316 and rs35086265) each being found once in a heterozygous state. The mutations and associated clinical phenotype features are shown in table 2.

The supporting evidence for the pathogenicity of the mutations is given in online supplementary table 2.

The remaining 111 patients included 36 with heterozygous non-synonymous nuclear variants of unknown significance (online supplementary table 3). No further testing for possible exon deletions or rearrangement on the other allele was performed.

NDUFB9 is a new complex I deficiency gene

Within our 152 subjects, we identified two rare sequence variants in the gene encoding the mitochondrial respiratory chain complex I subunit NDUFB9. Patient 33027 carried a single, heterozygous *NDUFB9* missense mutation (c.140G→T, p.Arg47Leu), with parents not available for genetic testing. Patient 35838 and his brother, 46986, harboured a homozygous missense mutation (c.191T→C, p.Leu64Pro), both parents being heterozygous carriers. Fibroblasts of patients 33027 and 35838 showed complex I activity as low as 21% and 39%, respectively, of the lowest control value. Both mutations affect amino acids conserved from human to *Drosophila*. To verify their pathogenicity, complementation experiments were performed. Expression of wild-type *NDUFB9* did rescue complex I activity up to low control values in fibroblasts from patient 35838 but not from patient 33027 (figure 2A). This result demonstrates that the homozygous missense mutation (c.191T→C, p.Leu64Pro) is indeed causative of complex I deficiency in subject 35838, whereas the heterozygous *NDUFB9* mutation (c.140G→T, p.Arg47Leu) is unlikely to have caused complex I deficiency in subject 33027. The sequence analysis of an affected sibling from patient 33027, who also displayed complex I deficiency, revealed that the heterozygous *NDUFB9* variant was absent. This finding argues for other causal mutations in this family.

Next, we investigated whether the mutation in *NDUFB9* was associated with a reduced amount of assembled complex I. To this end, we performed protein blot analysis of complex I subunits NDUFS1, NDUFS3, NDUFA9, NDUFB8 and NDUFB9

Table 1 Mitochondrial DNA-encoded likely pathogenic mutations identified in index patients with complex I deficiency

Patient ID	Gene	Variant	Heteroplasmy (%)	Reference	Associated clinical phenotype
33464	*ND3*	10158T→C p.Ser34Pro	<90	46	LS
35841	*ND3*	10191T→C p.Ser45Pro	>90	10	ESOC, LS-like, LS
33343	*ND3*	10197G→A p.Ala47Thr	>90	47	LS, D, S, LDYT
33456	*ND3*	10197G→A p.Ala47Thr	>90	47	LS, D, S, LDYT
33328	*ND5*	13042G→A p.Ala236Thr	>90	48	Optic neuropathy/retinopathy
44732	*ND6*	14459G→A p.Ala72Val	>90	49	LS
33346	*ND6*	14487T→C p.Met63Val	>90	50	LS, D, ataxia
33041	*MT-TL1*	3250T→C	>90	51	MM, CPEO
38807	*MT-TL1*	3302A→G	>90	52	MM
35799	*MT-TC*	5814T→C	>90	53	ME

CPEO, progressive external ophthalmoplegia; D, dystonia; ESOC, epilepsy, stroke-like episodes, optic atrophy, and cognitive decline; LDYT, Leber hereditary optic neuropathy and dystonia; LS, Leigh syndrome; ME, mitochondrial encephalopathy; MM, mitochondrial myopathy; S, stroke.

Table 2 Phenotypic features observed in complex I-deficient individuals with pathogenic nuclear DNA mutations

Clinical category	Feature	35834	49839	35836	35284	35817	33545	35830	33462	33006	35838	36179	33255	33460	35837	57122	35822	33354	33253	35797
Genetics	Mutations	c.130T →A het p.F44I; c.797G →A het p.R266Q	c.130T →A het p.F44I; c.797G →A het p.R266Q	c.797G →A het p.R266Q; c.1249C →T het p.R417C	c.976G →C het p.A326P; c.1594C →T het p.R532W	c.406C →T het p.R136W; c.615ins AGTG het p.A206 SfsX15	c.94G →C het p.G32R, X linked	c.296G →A hom p.G99E	c.9G →A hom p.W3X	c.23G →A hom p.G8D	c.191T →C hom p.L64 P	c.212T →A het p.V71D; c.384T →A het p.C128X	c.497G →A het p.G166E; c.683T →C het pV228A	c.683T →C het p.V228A; c.755A →G het p.D252G	c.1669C →T het p.R557X; c.1783A →G het p.T595A	c.1912 insA het p.638fsX642; c.2084A →G het p.Y695C	c.2083T →C het p.Y695H; c.2084A →G het p.Y695C	c.329A →T het p.D110V; c.968G →A het p.R323Q	c.316C →T hom p.R106X	c.352C →T hom p.Q118X
	Affected gene	*ACAD9*	*ACAD9*	*ACAD9*	*ACAD9*	*FOXRED1*	*NDUFA1*	*NDUFA10*	*NDUFAF2*	*NDUFAF4*	*NDUFB9*	*NDUFS1*	*NDUFS1*	*NDUFS1*	*NDUFS1*	*NDUFS1*	*NDUFS1*	*NDUFS2*	*NDUFS4*	*NDUFS6*
Cardiovascular	Cardiomyopathy hypertrophic	X	X	X	X	–	–	–	–	–	–	–	–	–	–	–	–	X	X	–
Metabolic	Raised blood lactate	X	X	X	X	–	X	X	X	X	X	X	–	X	X	X	X	X	X	X
	Raised CSF lactate				X	–	X	X	X	X	–	X	X	X	X	X	–	X	X	–
	CI activity	<25	<50	<25	<50	<25	<50	<25	<25	<25	>50	<50	>50	<50	<25	<50	<50	<25	<50	<50
Neurological	Leukodystrophy			–	–		X	–	X	X	–	–	X	X	X	X	X	–	–	–
	Lactate raised in brain [^{1}H]MR spectroscopy	–		–			X	X	X	X			X	X	X		X	X	X	X
	Loss of abilities	–	–	–	–	–	–	X	X	–	–	–	X	X	X	X	X	–	–	–
	Muscular hypotonia	X	–	–	–	–	X	X	–	X	X	X	X	X	X	X	–	–	X	X
	Psychomotor developmental delay	–	–	–	X	–	–	–	–	X	–	X	X	–	X	X	–	–	X	–
	Symmetrical basal ganglia lesions		–	–	–		X	X	–	X	–	–	–	–	–	X	–	–	X	–
	Symmetrical brainstem lesions		–	–	–		X	X	X	X	–	–	X	–	–	–	–	–	X	–
Miscellaneous	Failure to thrive	–	–	–	X	–	–	–	–	X		X	X	X	–	–	–	–	X	–
	AO (months)	<6	<6	<6	<6	<6	>6	>6	>6	<6	<6	<6	<6	<6	<6	<6	>6	<6	<6	<6
	Progressive course	X	–	X	X	X	–	X	X	X	X	X	X		X	X	X	X	X	X

The numbers in the table headings are ID numbers of the subjects.
X, feature present; –, within normal range/feature absent; empty box, information not available; AO, age of onset; CI, complex I; CSF, cerebrospinal fluid.

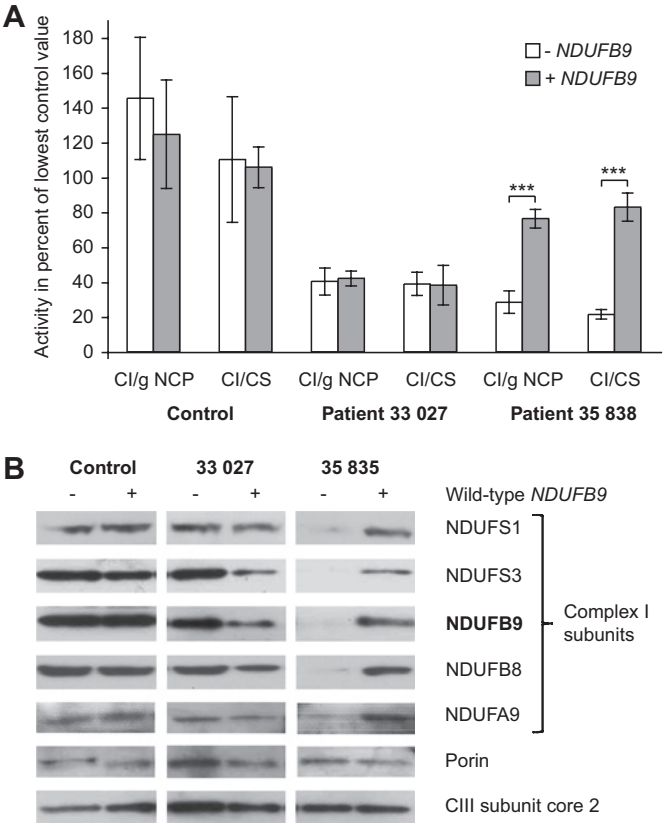

Figure 2 (A) Rescue of complex I defect by expression of wild-type *NDUFB9* cDNA in patient fibroblasts. Bar plots indicate complex I (CI) activity normalised by g of non-collagen protein (g NCP) or citrate synthase (CS), expressed as a percentage of the lowest control value ± SD. Activities were determined from cell lines transduced (+) or not transduced (−) with wild-type *NDUFB9* cDNA at three different time points. Lentiviral transduction with *NDUFB9* did not alter complex I activity in commercial control and patient 33027 cell lines, but did significantly increase complex I activity in fibroblasts from patient 35838. ***p<0.001. (B) NDUFB9 and complex I subunit protein expression with and without lentiviral expression of wild-type *NDUFB9* cDNA. Isolated mitochondria from control and patient fibroblasts, with (+) and without (−) lentiviral-mediated expression of wild-type *NDUFB9* cDNA, were analysed by immunoblotting with antibodies against mitochondrial complex I subunits (NDUFS1, NDUFS3, NDUFA9, NDUFB8 and NDUFB9). The core subunit 2 of complex III (CIII) and porin was used as a loading control. While the expression of wild-type NDUFB9 cDNA did not change the level of complex I (in relation to the loading controls) in fibroblasts from patient 33027, an increase in all investigated complex I subunits was found in patient 35838.

in mitochondria-enriched lysates from patient and control fibroblasts before and after lentiviral-mediated expression of a wild-type *NDUFB9* cDNA. Immunoblot patterns from fibroblast lysates of a control and subject 33027 were normal and did not change after lentiviral-mediated expression of wild-type *NDUFB9* cDNA. Contrariwise, immunoblot analysis of the cell lysate from patient 35838 showed a clear reduction in the mutant NDUFB9 subunit and the other investigated complex I subunits, NDUFS1, NDUFS3, NDUFB8 and NDUFA9 (figure 2B). The expression of wild-type *NDUFB9* cDNA resulted in a significant increase in NDUFB9 as well as all other investigated complex I subunits. These findings are concordant with the idea that NDUFB9 is necessary for assembly and/or stability of complex I.

Together, the complementation and immunoblot experiments provide evidence that *NDUFB9* is a new complex I deficiency-associated gene.

Mutational spectrum of complex I deficiency

Genetic diagnoses due to autosomal recessive (n=17), mtDNA (n=10) and X-chromosomal (n=1) mutations were established in 28 unrelated individuals. Of these mutations, 68% (19/28) were in genes encoding structural complex I subunits, 22% (6/28) affected genes coding for assembly factors, and 10% (3/28) were in mt-tRNA genes. Some 68% of the mutations in complex I subunits (14/19) affected highly conserved genes involved in redox activity (*NDUFS1* (n=6), *NDUFS2* (n=1)) or proton pump activity (*ND3* (n=4), *ND5* and *ND6* (n=2)).[3] In total, we found 35 unique mutations in 16 different genes, which highlights the extreme locus and allelic heterogeneity of mitochondrial complex I deficiency.

DISCUSSION

We report the results of a large-scale mutation screen using HRMA and Sanger sequencing of 74 candidate genes in 152 patients with complex I deficiency. To our knowledge, this study represents the largest cohort of patients studied so far. We identified the underlying molecular cause in 28 index patients and significantly increased the available phenotype data for nuclear complex I gene defects. For clinical classification and guidance of the diagnostic workup of complex I deficiency, genotype–phenotype correlations were sought. Although patients with mutations in the same gene have a large overlap in clinical features—for example, hypertrophic cardiomyopathy in patients with *ACAD9* mutation—this abnormality has also been reported in the *NDUFS2*, *NDUFS4*, *NDUSF8*, *NDUFA2* and *NDUFV2* genes. Furthermore, for many genes, only a few patients are described, which does not allow us to draw general conclusions. It is therefore all the more important that the molecular basis of more cases of complex I deficiency is available for the study of genotype–phenotype associations.

For the first time, we report a pathogenic mutation in the *NDUFB9* gene, encoding a complex I subunit. The causality of the newly identified missense mutation in *NDUFB9* was demonstrated by restoration of both amount and activity of complex I in patient-derived fibroblast cell lines after expression of wild-type *NDUFB9*. *NDUFB9* mutations are probably a rare cause of complex I deficiency, since they were not detected in an Australian cohort of 103 patients with complex I deficiency[37] and were present in only 1/152 patients of our cohort.

In our study, 10 patients harboured mtDNA mutations. As reported by others,[38 55] mtDNA mutations constitute a substantial fraction of complex I defects in children and even neonates. We conclude that sequence analysis of the entire mtDNA is mandatory in all cases of proven complex I deficiency.

High-throughput screening of complex I-related nuclear genes led us to identify 18 index patients with pathogenic mutations in nuclear-encoded genes. Most of the mutations were detected in known disease genes, with *NDUFS1* being the most commonly affected (six patients). However, after correction for the size of the open reading frame, *NDUFS1* cannot be considered a mutation hotspot.

Only 28/152 (18%) of our patients did obtain a molecular genetic definition. Reasons for this low diagnostic score may include (1) biased selection of patients, (2) high rate of false negatives, and, more interestingly, (3) the existence of a complex I-related protein set much larger than expected.

As for the first point, our cohort consisted of patients that were preselected for the most common mutations associated with complex I deficiency, implying that the estimated 30% of patients receiving a molecular diagnosis by routine genetic testing were a priori excluded. Furthermore, the inclusion criteria

Web resources

- The URLs for data presented herein are as follows:
- Guidelines mitochondrial disorders, http://www.aps-med.de/APS-P00.asp
- 1000 Genomes browser, http://browser.1000genomes.org/
- MITOMAP, http://www.mitomap.org/MITOMAP
- Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/

for our cohort were kept wide since our aim was to test all patients with a biochemical phenotype showing complex I as the only or most prominent enzyme defect. Nevertheless, a total of 95 patients fulfilled the criteria for a 'definite' diagnosis of isolated complex I deficiency as defined by Kirby *et al*.[56] Thereof, 62 patients had a residual complex I activity corrected for citrate synthase of less than 25% of the mean. Forty-seven patients had a residual activity of 25–40%. Thirty-three of them had (1) similar activity in multiple tissues, cell lines or siblings or (2) supportive findings from histology or electron microscopy. In the remaining subjects, residual complex I activity ranged from 40% to 68%, being included essentially because of suggestive clinical presentation. Retrospectively, a molecular diagnosis was found in 22/95 patients with a definite diagnosis of complex I deficiency (23%). However, six patients with either a residual complex I activity >40% of the mean (n=5) or an activity of 25–40% but no additional evidence of a diagnosis of complex I deficiency (n=1) would have been excluded from the screening by using a more stringent biochemical cut-off, including the patient with a functionally confirmed pathogenic *NDUFB9* mutation. These findings demonstrate that phenotypic stratification increases the diagnostic score (23% with a definite diagnosis vs 11% with less stringent diagnostic criteria) at the cost of a higher false negative rate.

As for the second point, it is possible that, in a fraction of patients, the causal mutations were not detected because of reduced sensitivity of the screening based on DNA melting profiles, especially for homozygous variants, or because ~7% of some exon sequences failed to be PCR-amplified and were not further analysed. Furthermore, three genes (*C20orf7*, *C8orf38* and *NDUFAF3*) reported to be involved in complex I functioning were not investigated. However, the fraction of resolved cases, as well as the sensitivity of the screening approach, is consistent with recently reported results from a screen of 103 candidate genes in 60 unsolved patients with complex I deficiency,[37] which established new genetic diagnoses in 11 index cases (19%).

As for the third point, the formation and function of complex I are finely tuned, complicated processes that are likely to require additional, unknown factors for assembly, turnover, and activity control of the complex. The large number of patients who failed to obtain a molecular diagnosis in candidate gene approaches supports this view.

In conclusion, by implementing an ad hoc, large-scale genetic screen, we identified pathogenic mutations in 28 patients with complex I deficiency. Our study led to the identification of an *NDUFB9* mutation as a new cause of complex I deficiency. Although in some of the undiagnosed individuals we detected potentially causative variants that may contribute to complex I deficiency, about half of the patients carried no rare variants. More exhaustive screening strategies, such as exome sequencing coupled with functional validation,[42] will be needed to pinpoint the molecular genetic cause in these remaining individuals. The identification of new mutations and disease genes associated with complex I deficiency will allow investigators to gain new insights into the function of complex I, and help clinicians to offer better genetic counselling, improve patient management, and develop more effective therapies.

Author affiliations
[1]Institute of Human Genetics, Technische Universität München, Munich, Germany
[2]Institute of Human Genetics, Helmholtz Zentrum München, German Research Center for Environmental Health, Neuherberg, Germany
[3]Unit of Molecular Neurogenetics, Neurological Institute 'Carlo Besta'-IRCCS Foundation, Milan, Italy
[4]Department of Pediatrics, Paracelsus Medical University, Salzburg, Austria
[5]Städtisches Klinikum München GmbH, Department Klinische Chemie, Munich, Germany
[6]Institut National de la Santé et de la Recherche Médicale (INSERM), Paris, France
[7]Institute of Genetic Medicine, Newcastle University, Newcastle upon Tyne, UK
[8]Department of Pediatrics and Adolescent Medicine, First Faculty of Medicine, Charles University in Prague and General University Hospital in Prague, Prague, Czech Republic
[9]Department of Pediatrics, University Hospital Center Zagreb and School of Medicine, Zagreb, Croatia
[10]Unit of Child Neurology, Neurological Institute 'Carlo Besta'-IRCCS Foundation, Milan, Italy
[11]Department of Paediatrics, Technische Universität München, Munich, Germany

Acknowledgements We are grateful to the patients and their families for their participation, and especially to R Hellinger and A Löschner for their technical support.

Funding TM and HP were supported by the Impulse and Networking Fund of the Helmholtz Association in the framework of the Helmholtz Alliance for Mental Health in an Ageing Society (HA-215) and the German Federal Ministry of Education and Research (BMBF) funded German Center for Diabetes Research (DZD e.V.) and Systems Biology of Metabotypes grant (SysMBo #0315494A). EL, FI, TM, MZ and HP were supported by the grant RF-INN-2007-634163 from the Italian Ministry of Health. TM, PF and HP were supported by the BMBF funded German Network for Mitochondrial Disorders (mitoNET #01GM0867). EL, FI and MZ were supported by the Pierfranco and Luisa Mariani Foundation Italy, Fondazione Telethon Italy grants number GGP07019 and GGPP10005, Fondazione MitoCon-ONLUS, Fondazione Giuseppe Tomasello—ONLUS, and grant RF-INN-2007-634163. JAM and WS were supported by the Österreichische Nationalbank-Jubiläumsfonds grant number 12568 and the Vereinigung zur Pädiatrischen Forschung und Fortbildung Salzburg.

Competing interests None.

Patient consent Obtained.

Ethics approval Ethics committee, Faculty of Medicine, Technical University of Munich.

Contributors HP designed the study; MH, EL, FI, JK, JM, UA, VT, AR, RH, MT, IB, GU, BR, WS, MZ and PF collected clinical and biochemical data; TH, MH and HP analysed and interpreted the data; TH, FM, MF, RD, IH and HP performed mutation screening; KD, BH and AI conducted complementation experiments; TH, EL, TM, MZ and HP wrote the manuscript; and all authors critically revised the manuscript and approved the version to be published.

Provenance and peer review Not commissioned; externally peer reviewed.

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J Med Genet 2012 49: 83-89 originally published online December 26, 2011
doi: 10.1136/jmedgenet-2011-100577