Molecular diagnostics of mitochondrial disorders

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Received 7 June 2004; received in revised form 20 July 2004; accepted 26 July 2004
Available online 11 August 2004

Abstract

The mitochondrial respiratory chain (RC) results from the expression of both mitochondrial and nuclear genes. The number of disease-causing mutations in nuclear genes is steadily growing and mitochondrial DNA (mtDNA) deletions and mutations account for no more than 15–20% of pediatric patients. Unfortunately, the disease-causing mutations have been identified for only a small number of patients. Thus, elucidating the genetic bases of RC is both essential for genetic diagnosis of patients and for fundamental knowledge of these disorders. The molecular diagnostics of mitochondrial disorders come under both genetic diagnosis and research. Indeed, identification of a new gene in a specific patient allows to perform genetic diagnosis in other families and identification of mutations in already known disease-causing genes allows to constitute a cohort of patients for further functional studies. Thus, elucidating the genetic bases of RC deficiency is an essential task that needs the use of several appropriate strategies. Fine phenotype of patients and candidate gene screening is the first step for the constitution of a well-characterized cohort of patients. Genetic mapping has to be used in large families. This approach is greatly enhanced in the case of consanguineous families. The consanguinity of the parents should also lead to test genetic markers surrounding the gene loci rather than to directly sequence several candidate genes. However, the main problem is encountered in the cases of sporadic cases for which no genetic approaches can be developed. In these cases, functional complementation by human chromosomes or cDNA is the only presently available strategy.

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Keywords: mtDNA; Nuclear genes; Molecular diagnosis

1. Introduction

Mitochondrial disorders are highly heterogeneous diseases. Indeed, if the common point is a respiratory chain (RC) deficiency, these diseases are in fact due to different enzyme deficiencies. Moreover, the clinical presentation of the patients is very heterogeneous. The age of onset is variable and the deficiency can result in an isolated organ deficiency or a multivisceral involvement. Finally, a very large number of genes are involved in RC function and these diseases can follow any mode of inheritance. Metabolic and enzymological studies are essential for the diagnosis of RC deficiency but they are only indicative for the genetic and molecular diagnosis. Therefore, one of the main challenges in this field is not only to identify new genes responsible for these deficiencies but also to decide what gene has to be screen in patients.

2. Primary or secondary RC deficiency

Mitochondria have a central role in the cell and numerous functions and metabolic pathways are completely or partially associated with this compartment. These functions and pathways are more or less linked to the RC and one can hypothesize that a deficiency of one of them can lead to a secondary RC deficiency. This is for example the case in Friedreich ataxia where mutations in frataxin, a
protein involved in iron–sulfur metabolism, result in a RC deficiency [1]. This doesn’t rule out the diagnosis of RC deficiency in the disease but should be borne in mind for the molecular diagnosis.

3. Mitochondrial or nuclear genes?

All RC complexes, except complex II, include subunits encoded both by the mitochondrial and the nuclear genomes. Thus, in all deficiencies, except isolated complex II deficiency, the underlying mutation can affect either a mitochondrial or a nuclear gene. Until now, the enzymological study by itself cannot help to settle between a mitochondrial or a nuclear gene. However, at least in pediatric patients, the sporadic nature of the disease or the small size of the families precludes this kind of information. Nuclear or mitochondrial origin of the deficiencies can be determined by fusing patient fibroblasts with mtDNA-less (mitochondrial origin of the deficiencies can be determined families precludes this kind of information. Nuclear or mitochondrial origin of the deficiencies can be determined by fusing patient fibroblasts with mtDNA-less (mitochondrial origin of the deficiencies can be determined).

6. Complex I deficiencies

Complex I is composed of a very large number of subunits encoded by mtDNA and nuclear genes. The first mutations of isolated complex I deficiencies have been identified by the group of Jan Smeitink in Netherlands [8]. This pioneer work prompted us to perform a mutational analysis study of several nuclear genes of complex I by D-HPLC as well as mitochondrial genes by direct sequencing.

6.1. Mitochondrial complex I gene screening

Seven complex I subunits are encoded by the mitochondrial genome. These genes comprise 296 bp to 3.9 kb, theoretically allowing an easy and fast study. However, several points hamper a rapid screening of these genes.

mtDNA is known to present a high number of polymorphic nucleotide substitutions. Therefore, all indirect techniques aimed to detect heteroduplexes will detect a large number of abnormal patterns. Sequencing of the corresponding regions usually reveals that these abnormal patterns are actually due to one or more polymorphisms. Thus, indirect approaches such as denaturing gradient gel electrophoresis (DGGE), single strand conformation polymorphism (SSCP) or denaturing high-performance liquid chromatography (DHPLC) are difficult to use in the screening of mitochondrial genes when looking for unknown mutations. These approaches are also difficult for the screening of a specific mutation in new patients who can carry yet undetected polymorphism(s). Direct sequencing of those genes is one of the more appropriate ways to study them.

A well-known feature of mtDNA mutations is that they are most often heteroplasmic, the ratio between normal and mutant mtDNA in a specific tissue being related to the severity of the disease and to the RC deficiency. Organs or tissues with high level of mutant mtDNA express the RC deficiency, whereas those with lower amounts of mtDNA don’t. Direct sequencing is the only relevant approach but it is a non-quantitative technique that, in some cases, doesn’t detect heteroplasmic substitutions when lower than 40%. For this reason, study of mitochondrial genes absolutely requires work on the affected tissue(s), which is often in very low amounts after having used it for RC
enzymological study, molecular screening of common mitochondrial mutations or studying other candidate nuclear genes.

Direct sequencing of mitochondrial complex I genes has been performed in a series of patients with isolated complex I deficiency irrespective of any information deduced from the pedigree. This allowed to identify mutations in ND1, ND3, ND5 and ND6 genes in 12 patients. It is interesting to note that these mutations often involve thymine to cytosine transitions [9,10,2]. Screening the mitochondrial complex I genes resulted in an unexpectedly high detection mutation rate in our series (20% of our complex I-deficient children, Table 1).

6.2. Nuclear complex I gene screening

We studied the most conserved complex I nuclear genes that represent the catalytic activity of the complex. This study was performed on total RNA extracted from patient fibroblasts and reverse transcribed. The coding sequences were then amplified in overlapping fragments and the PCR fragments were submitted to D-HPLC analysis, which allows to detect DNA heteroduplexes by ion-pair reverse phase HPLC under partially denaturing conditions [11]. Abnormal fragments were then directly sequenced.

Several pitfalls can be encountered when studying these genes, as well as other nuclear genes. DHPLC can sometimes detect only one abnormal profile corresponding to a heterozygous mutation. Direct sequencing of the complete coding sequence has to be performed to identify the second mutation which was undetected by the DHPLC, whereas several conditions of temperature or elution of the DNA have been used. It is often frequent to detect only one apparently homozygous mutation when working on cDNA, whereas parents were non-consanguineous. This is sometimes related to the presence of a non-sense mutation on the second allele, which results in an instable transcript. Conversely, an apparent homozygosity when studying genomic DNA can be due to a large deletion of the second allele. Thus, the segregation of the mutation in the family has to be followed to avoid false diagnostics.

The different nuclear gene mutations identified in patients with isolated complex I deficiency are mainly associated with Leigh or Leigh-like disease. But mutations in several complex I nuclear genes have been excluded in numerous patients with Leigh or Leigh-like disease. Some mutations give rise to other clinical presentation, cardiomyopathy and encephalopathy for NDUFV2 [12] or NDUFS4 [13] mutations for example. This can help for deciding which gene has to be first studied in a new patient. However, NDUFS4 mutations are also known to give various clinical presentations again hampering any genotype–phenotype correlation [13].

Screening the mitochondrial complex I genes resulted in a detection mutation rate in our series of approximately 20% (Table 1).

7. Complex IV deficiencies

Complex IV deficiencies present the same difficulties as complex I as this complex is composed of several subunits from mitochondrial and nuclear origin. Moreover, the clinical presentation of the patients is also very variable. An additional difficulty is related to the low number of COX subunit gene mutations. Several groups have screened the three mitochondrial and the ten nuclear genes encoding COX subunits, but few mutations in mitochondrial COX genes have been reported [2] and no nuclear COX gene mutation is presently known. However, it clearly appears that complex IV deficiencies are most often due to assembly gene mutations. This suggests that the prevalent non-maternally transmitted mutations causing COX deficiency are present in genes involved in COX assembly.

SURF1 represents a major gene for Leigh syndrome associated with COX deficiency as 25–75% of Leigh–COX− patients had SURF1 mutations [14]. Therefore, this gene has to be studied in Leigh–COX− patients. But, we
and others have reported SURF1 mutations in patients with completely different disease such as villous atrophy or leukodystrophy [6,7]. Therefore, a systematic study of SURF1 gene in all patients with COX deficiency has to be considered.

Mutations in SCO2 gene have been identified in several patients with cardioencephalomyopathy [5]. Exclusion of SCO2 mutations in patients with cardioencephalomyopathy as well as SCO2 mutations associated with other clinical presentations has not been yet reported. Therefore, this gene remains the only candidate gene for this disease.

Four other COX assembly genes have been shown to be responsible for COX deficiencies. Mutations in COX10 gene, encoding a heme A farnesyl transferase, an enzyme required for the correct maturation of heme of the cytochrome c oxidase, have been first identified in a patient with tubulopathy and leukodystrophy [15]. However, mutations of this gene in two other patients give rise to anemia and Leigh Syndrome, or anemia, sensorineural deafness, and fatal infantile hypertrophic cardiomyopathy [16]. Mutations in SCO1 [17] involved in mitochondrial copper metabolism give rise to hepato- and ketoacidotic coma, but has been reported in only one family. Mutations in COX15 gene, which is necessary for the final step in heme A biosynthesis, led to hypertrophic cardiomyopathy [18]. Finally, mutations in LRPPRC cause Leigh syndrome-French–Canadian type [19]. This syndrome is yet restricted to geographically isolated Canadian families originating from a common ancestor.

Mutations in these four last genes correspond, according to the literature, to very few patients. Complex IV deficiency represents around 20% of RC disorders, which hardly suggests that numerous other genes are responsible of these diseases and have to be identified. Therefore, for most isolated complex IV deficiencies, there is no definite strategy for molecular diagnosis and the main conclusion drawn from the previous studies is that a lot of nuclear genes have to be screened whatever the clinical presentation of the patients is. In order to avoid a tedious and expansive study of all the possible candidate genes, we took advantage of the consanguinity of some families in our series of isolated COX deficiencies. Indeed, the high rate of parental consanguinity and recurrence of the disease in COX-deficient families of northern and western African ancestry is highly suggestive of an autosomal recessive genotype. We therefore hypothesized that the disease-causing gene is a nuclear one, which is inherited from a common ancestor. In this case, the patients are homozygous for the mutant allele, whereas the parents are heterozygous. Healthy sibs are either wild-type homozygous or heterozygous. Microsatellite markers closely surrounding (1–2 Mb) several candidate genes were then studied. Heterozygosity of the markers excluded the corresponding gene as disease-causing, whereas homozygosity represented a clue for studying it. The same approach also has to be performed in multiplex non-consanguineous families. Haploidentity of the affected sibs for the markers surrounding a candidate gene should prompt the study of this gene. Availability of all members of the family is helpful for this kind of study, but this indirect screening of candidate genes can be used even if only the DNA of patients is available. In this case, studying several markers at each locus can compensate the absence of relatives’ DNA.

With this approach, we excluded all known COX subunits and assembly genes in a series of 20 patients from multiplex or consanguineous families (Table 2).

### 8. Complex I+IV deficiencies/multiple deficiencies

Multiple deficiencies can result from a large panel of gene mutations: large mtDNA deletions, mtDNA point mutations of genes involved in mitochondrial translation, mutations in mtDNA metabolism nuclear genes, mutations in RC assembly genes.

In adult patients, the concomitant analysis of the clinical presentation and the pedigree of the family is often helpful for determining the gene to be studied. Indeed, a maternal transmission of the trait associated with diabetes and
deafness±renal disorder or cardiac involvement is suggestive of the A3243G MELAS mutation [2]. Conversely, an autosomal dominant transmission of the trait associated with ptosis, ophthalmoplegia and muscle weakness is highly evocative of POLG, ANT1 or Twinkle mutation [20–22]. However, pedigrees are often non-informative especially in pediatric patients that hamper a clear-cut diagnostic strategy.

Deoxyguanosine kinase (DGUOK) and thymidine kinase (TK2) mutations have been reported in association with complex I+IV deficiency in patients presenting hepatocerebral and myopathic forms of mtDNA depletion syndrome, respectively [23,24]. Considering the high number of patients with hepatic and neurological disease in our series of complex I+IV deficiency, we decided to test the DGUOK gene irrespective of the presence of mtDNA depletion. DGUOK was tested by using microsatellite markers surrounding DGUOK gene on chromosome 2p13 for patients from consanguineous parents, or by direct sequencing of the exons for non-consanguineous families. Among these 20 patients, we identified either one homozygous or two heterozygous mutations for only four of them. Moreover, 14 other patients with different clinical presentations did not present any DGUOK mutation.

9. Quinone deficiencies

Quinone deficiencies represent a rare cause of mitochondrial diseases. They have to be suspected when quinone-dependent activities (complex I+III, complex II+III) are in the lowest values, whereas isolated complexes present normal activities. Direct measurement of coenzyme Q_{10} will confirm the deficiency. The biosynthesis of ubiquinone has been extensively studied in bacteria and yeast and is common to the mevalonate pathway [25]. The human genes encoding the various enzymes of this pathway have been deduced from the data obtained in yeast and bacteria. Identification of the mutations responsible for these deficiencies can be performed either by genetic mapping in large families or by testing candidate genes in sporadic cases and small families. Once again, these genes can be indirectly tested, in the case of multiplex or consanguineous families, by the use of microsatellite markers surrounding the candidate genes.

10. Complex II deficiencies

These deficiencies represent 4% of mitochondrial diseases. Complex II is composed of four subunits all nuclearly encoded. Until now, only mutations in the SDH1 gene have been identified in patients with isolated complex II deficiency and Leigh syndrome [26]. No complex II assembly protein is known in human. Therefore, molecular screening of these deficiencies consists only to study the four genes encoding subunits of complex II.

11. Complex III deficiencies

Complex III (CIII) deficiency represents a relatively rare cause of respiratory enzyme dysfunction. Indeed, in our experience, among all RC enzyme-deficient patients, only 7% had a CIII deficiency. Moreover, the clinical presentation of complex III-deficient patients is very heterogeneous. Cytochrome b is the only subunit of complex III that is encoded by mtDNA. Until now several cyt b mutations have been described in association with various clinical presentations. Interestingly, most of the patients presented with the predominant feature of severe exercise intolerance, sometimes including muscle weakness and/or myoglobinuria. Other presented cardiomyopathy or MELAS and encephalomyopathy [27]. Only one mutation in a nuclear gene encoding a complex III subunit has been identified by systematic analysis of all complex III subunit genes [28]. Finally, BCS1 gene mutations have been identified in patients presenting tubulopathy, encephalopathy and liver failure [29]. Therefore, molecular diagnosis of these deficiencies should include mitochondrial cyt b and BCS1 genes sequencing in patients with exercise intolerance and renal, neurological and hepatic involvement.

12. Complex V deficiencies

The main mutation associated with complex V deficiency is T>C8993 in the mitochondrial ATP6 gene. This mutation can result either in a Leigh disease syndrome or in a neurogenic muscle weakness with ataxia, and retinitis pigmentosa (NARP) [27]. This mutation is currently detected by PCR-digestion.

13. Prenatal diagnosis of mtDNA mutation

Prenatal diagnosis of mtDNA point mutations is a difficult task due to the precise estimation of heteroplasmy and the uncertainty about its effects. Estimation of heteroplasmy is usually performed by PCR-digestion and various techniques have been developed in this field. Most of them consist of the addition of labeled dNTP in the last cycles of the PCR. In order to improve this detection, we have set up a new approach by performing only 22 cycles of PCR with a fluorescent-labeled oligonucleotide followed by a classical restriction and migration of the PCR product on a sequencing machine allowing to reliably estimate the ratio between normal and mutant mtDNA [30]. The reliability of this technique which can be performed on
small amounts DNA, and even on a single cell, is promising for prenatal diagnosis or pre-implantation genetic diagnosis.

Prenatal diagnosis of mtDNA NARP and MELAS mutations can be performed in at-risk families by the above-described technique. Because it is not possible to predict with confidence the way in which heteroplasmic mtDNA mutations segregate within tissues, and find clinical expression, we usually perform several estimations during pregnancy.

14. Conclusions

Despite the growing identification of genes of mitochondrial disorders, molecular diagnosis remains always difficult. This is mainly due to the double genetic origin of RC proteins, the great heterogeneity of the clinical presentation of the patients and the inconstant genotype–phenotype correlations. The molecular screening of complex I deficiencies only give a satisfactory rate of mutation detection as mutations in either mitochondrial or nuclear genes encoding complex I subunits can be identified in 30–40% of the patients. For other RC deficiencies, the molecular diagnosis consists most often of an exclusion diagnosis, which is in turn a prerequisite for research works aimed to identify the disease-causing genes.

References


