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Isolated deficiencies of OXPHOS complexes I and IV are identified accurately and quickly by simple enzyme activity immunocapture assays

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ABSTRACT

OXPHOS deficits are associated with most reported cases of inherited, degenerative and acquired mitochondrial disease. Traditional methods of measuring OXPHOS activities in patients provide valuable clinical information but require fifty to hundreds of milligrams of biopsy tissue samples in order to isolate mitochondria for analysis. We have worked to develop assays that require less sample and here report novel immunocapture assays (lateral flow dipstick immunoassays) to determine the activities of complexes I and IV, which are far and away the most commonly affected complexes in the class of OXPHOS diseases. These assays are extremely simple to perform, rapid (1–1.5 h) and reproducible with low intra-assay and inter-assay coefficients of variability (CVs) s (<10%). Importantly, there is no need to purify mitochondria as crude extracts of whole cells or tissues are suitable samples. Therefore, the assays allow use of samples obtained non-invasively such as cheek swabs and whole blood, which are not amenable to traditional mitochondrial purification and OXPHOS enzyme analysis. As a first step to assess clinical utility of these novel assays, they were used to screen a panel of cultured fibroblasts derived from patients with isolated deficiencies in complex I or IV caused by identified genetic defects. All patients (5/5) with isolated complex IV deficiencies were identified in this population. Similarly, almost all (22/24) patients with isolated complex I deficiencies were identified. We believe that this assay approach should find widespread utility in initial screening of patients suspected of having mitochondrial disease.

1. Introduction

Defects of the oxidative phosphorylation (OXPHOS) system are the cause of many different diseases such as Leigh's, MERRF, MELAS, NARP, and LHON, each individually rare, but with a combined incidence of about 1:5000 [1–3]. There are 5 large complexes involved in OXPHOS, namely NADH ubiquinone oxidoreductase or complex I, succinate ubiquinone oxidoreductase (complex II), ubiquinone cytochrome *c* oxidoreductase (complex III), cytochrome *c* oxidase (complex IV) and the ATP synthase (complex V). Isolated deficiencies in complex I (CI) are the most common followed by isolated deficiencies of complex IV (CIV) [4–7]. There are also genetic defects in which multiple OXPHOS complexes including CI and CIV are deficient resulting either from long deletions in the mtDNA, from point mutations in the several tRNAs in this genome required for protein synthesis within the organelle and in nuclear genes involved e.g. in mitochondrial maintenance and the mtDNA translation machinery.

Both CI and CIV are large multiprotein enzyme complexes composed of both mtDNA-encoded and nuclear DNA encoded subunits. Therefore, deficits in amount or enzyme functioning can result not only from mutations or damage to nuclear or mtDNA-encoded structural proteins, but also from alterations in nuclear DNA-encoded factors required for proper assembly of these enzyme complexes. Indeed, it has been reported that most cases of early-onset, inherited isolated CI and CIV deficiencies are caused by defects in complex-specific assembly factors and not in complex-specific structural proteins [8].

Detection and diagnosis of OXPHOS deficiencies has until now depended on an extensive evaluation of clinical presentation of patients along with blood and urine chemistry analysis, particularly for lactic acid levels and alanine and unexpected metabolic intermediates. Also done are activity measurements of the 5 OXPHOS complexes, usually from large biopsy of muscle or from fibroblasts taken from the patient and cultured to produce workable amounts for mitochondrial purification. Open biopsy is invasive, particularly in infants presenting with potential OXPHOS disease and requires general anesthesia [9]. More acceptable would be methods for measuring OXPHOS activities that do not require mitochondrial isolation and could be performed on samples obtained by non-invasive means.

We have recently developed a set of assays for determining the amount and activity of OXPHOS complexes that use monoclonal antibody immunocapture to isolate each complex for analysis. Several attributes provide these assays with the potential to be used in primary screening of patients with suspected OXPHOS deficiency. The

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assays are rapid, accurate and can be done on a few micrograms of cell extract. Therefore they can be done on samples obtained by much less invasive means than an open biopsy e.g. cheek swabs, urine sediment or blood. If a biopsy is necessary, for example because the defect is localized to muscle, needle biopsy provides sufficient material for analysis.

As a first step to evaluate the utility of these novel methods we have compared results obtained by the immunocapture assay approach with the previous gold standard assays obtained using intact mitochondria. To do this we have taken advantage of an important resource, a set of fibroblast cell lines from patients with defined OXPHOS deficiencies collected over several years by the team of excellent clinical investigators at the Nijmegen Centre for Mitochondrial Disorders, Radboud University Nijmegen Medical Centre, Netherlands. For each of these cell lines a clinical profile of the patient is available as well as OXPHOS activities measured spectrophotometrically on mitochondria isolated from each. We show that the dipstick assays work well to identify CIV and CI patients from controls in almost all (27 of 29) patients tested. There are 2 exceptions, which are discussed in detail later.

2. Materials and methods

2.1. Patients

The clinical, biochemical and genetic data from analysis of the patients' cultured fibroblasts is presented in Table 1. The study group was composed of 24 patients with isolated complex I deficiencies (values below the lowest control), 5 with isolated complex IV deficiencies (values below the lowest control) and 10 age-matched controls. Complex I and IV activities were measured as previously described [10,11].

Table 1
Patient data information

Patient #	Gene	Mutation	CI activity (mU/UCOX)	CIV activity (mU/UCS)
Complex I				
1	NDUFS2	P299Q	29	597
2	NDUFS2	S413P	44	2189
3	NDUFS2	R228Q	54	1163
4	NDUFS2	R228Q	nd	860
5	NDUFS2	nd	29	958
6	NDUFS2	F84L/E104G	71	650
7	NDUFS4	W97X	nd	nd
8	NDUFS4	W97X	65	681
9	NDUFS4	R106X	60	nd
10	NDUFS4	R106X	36	860
11	NDUFS4	VPEEH167/VEKSIstop	62	783
12	NDUFS4	K158fs	75	nd
13	NDUFS7	V122M	65	710
14	NDUFS8	P79L/R102H	80	870
15	NDUFS8	R94C	16	704
16	NDUFV1	R59X/T423M	70	1350
17	NDUFV1	A341V	50	1100
18	NDUFV1	A211V	nd	nd
19	NDUFV1	R59X/T423M	80	800
20	ND2	L71P	42	920
21	ND3	10191T>C	59	927
22	ND6	M63V	67	924
23	ND5	13513G>A	93	1174
24	ND5	13513G>A	78	1154
Complex IV				
25	SURF1	121	117	
26	SURF1	G124R	nd	<62
27	SURF1	INS470A	243	144
28	SURF1	326insATdelTCTGCCAGCC	133	104
29	COX10	M1T	169	220
Controls				
Mean			140 ($n=10$)	805 ($n=6$)
Range			114–190	620–1077

nd = not determined.

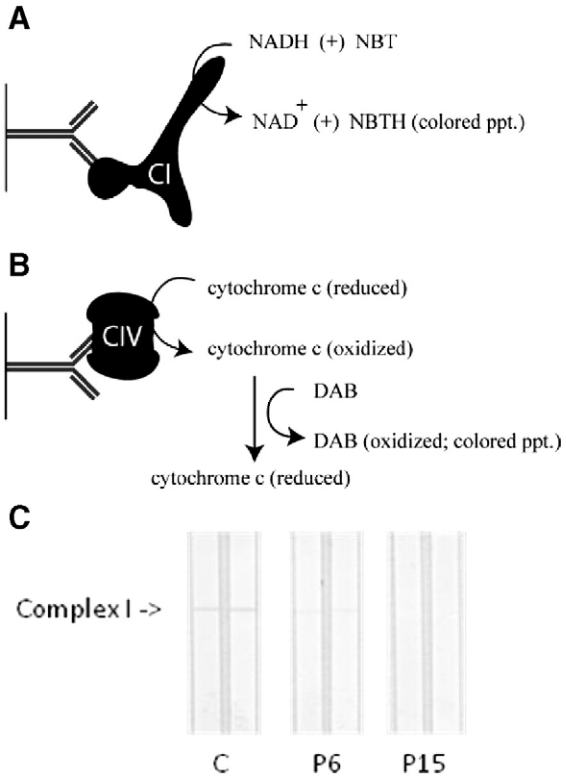

Fig. 1. Complex I and IV enzyme activity dipstick assays. (A) Complex I (CI) Activity dipstick reaction. Immunocaptured CI oxidizes NADH and reduces NBT, which forms a colored precipitate at the complex I immunocapture zone. The signal intensity is proportional to the concentration of enzymatically active complex I in the sample. (B) Complex IV (CIV) Activity dipstick reaction. Immunocaptured CIV oxidizes cytochrome *c*, which subsequently oxidizes DAB to form a colored precipitate at the complex IV immunocapture zone. The signal intensity is proportional to the concentration of enzymatically active CIV in the sample. (C) Representative images of CI activity dipsticks run (in duplicate) using samples taken from control fibroblasts (C) and patients with either partial (P6) or complete (P15) reduction in CI activity.

2.2. Whole cell protein extract preparations

Frozen fibroblasts cell pellets were thawed on ice and washed three times with 500 µl of PBS (pH 7.4). The cell pellet was then solubilized in 300 µl of Extraction buffer (1.5% n-Dodecyl-β-D-maltopyranoside (LM) (Anatrace, Maumee, OH), 25 mM Hepes (Sigma, St. Louis, MO) 100 mM NaCl (Sigma) with protease inhibitors (0.5 µg/ml leupeptin, 0.5 µg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride) for 20 min on ice. Following solubilization, the extract was clarified by centrifugation at 16,000 rpm for 20 min at 4 °C, the supernatant saved and stored frozen at −80 °C. Protein concentrations of the solubilized samples were determined by BCA analysis (Pierce, Rockford Ill.).

2.3. Monoclonal antibodies

Monoclonal antibodies (mAbs) that recognize native complex I were made by immunizing mice with sucrose gradient purified bovine heart complex I while anti-complex IV mAbs were made by immunizing mice with purified bovine heart mitochondria. The procedures for hybridoma preparation were as previously described [12], and the resulting hybridomas were screened for secretion of mAbs that specifically immunoprecipitate either complex I (mAb 18G12BC2) or complex IV (mAb 31E91B8) from both human and bovine heart mitochondria. The resulting immunoprecipitates were