Original Article

Non-Invasive Screening of Cytochrome c Oxidase Deficiency in Children Using a Dipstick Immunocapture Assay

(immunocapture assay / SURF1 / Cytochrome c oxidase deficiency / Leigh syndrome / non-invasive diagnostics)

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Abstract. Cytochrome c oxidase (CIV) deficiency is among the most common childhood mitochondrial disorders. The diagnosis of this deficiency is complex, and muscle biopsy is used as the gold standard of diagnosis. Our aim was to minimize the patient burden and to test the use of a dipstick immunocapture assay (DIA) to determine the amount of CIV in non-invasively obtained buccal epithelial cells. Buccal smears were obtained from five children with Leigh syndrome including three children exhibiting a previously confirmed CIV deficiency in muscle and fibroblasts and two children who were clinical suspects for CIV deficiency; the smear samples were analysed using CI and CIV human protein quantity dipstick assay kits. Samples from five children of similar age and five adults were used as controls. Analysis of the controls demonstrated that only samples of buccal cells that were frozen for a maximum of 4 h after collection provide accurate results. All three patients with confirmed CIV deficiency due to mutations in the SURF1 gene exhibited significantly lower amounts of CIV than the similarly aged controls; significantly lower amounts were also observed in two new patients, for whom later molecular analy-

sis also confirmed pathologic mutations in the SURF1 gene. We conclude that DIA is a simple, fast and sensitive method for the determination of CIV in buccal cells and is suitable for the screening of CIV deficiency in non-invasively obtained material from children who are suspected of having mitochondrial disease.

Introduction

Leigh syndrome (LS) is among the most common mitochondrial disorders in childhood, especially in the Slavonic population (Bohm et al., 2006). Clinically, LS is characterized as a progressive neurodegenerative disease exhibiting symmetric necrotizing lesions in the basal ganglia, thalamus and brainstem. Early postnatal adaptation in affected children is usually uneventful, but hypotonia, failure to thrive, frequent attacks of irregular breathing, developmental delay and hyperlactacidemia usually appear between the ages of 6 and 18 months; an attenuated course of the disease has also been described in children (Piekutowska-Abramczuk et al., 2009). The genetic background of LS is heterogeneous but is most commonly associated with cytochrome c oxidase (the respiratory chain complexes IV (CIV) of the oxidative phosphorylation system (OXPHOS)) deficiency, which is associated with mutations in the SURF1 gene (which encodes the Surf1 protein). Surf1 plays a key role in the early stages of the CIV assembly process (Yao and Shoubridge, 1999). Loss of Surf1 function results in decreased amounts of fully assembled CIV, thus extensively affecting the function of the entire OXPHOS (Williams et al., 2004).

Due to the high variability of symptoms, mitochondrial diagnosis is a complex and difficult process that combines biochemical analyses and molecular genetic methods. The traditional approach used to diagnose mitochondrial disorders usually requires biochemical analyses of invasively obtained muscle biopsy samples, a procedure that is highly stressful for small children. To minimize the burden on the patients, it is possible to analyse the entire spectrum of potential genes using
next-generation sequencing methods. It would be highly advantageous if some of the needed biochemical analyses could be performed with tissues obtained using non-invasive methods. Recently, epithelial cells obtained from buccal smears have been shown to represent suitable material for analysing respiratory chain complexes I (Cl) and CIV using dipstick immunocapture assays (DIA, ab109722, ab109877, MitoSciences, Abcam*, Cambridge, UK). In a study with 40 patients who were clinically suspect of having mitochondrial disease due to CI or CIV deficiency, biochemical analyses of samples obtained using muscle biopsy and samples of buccal cells were found to provide similar results in 80% of patients (Goldenthal et al., 2011). The aims of our study were to minimize the patient burden and to test the efficacy of DIA for the diagnosis of CIV deficiency in non-invasively obtained buccal epithelial cells.

**Material and Methods**

**Patients**

Five patients aged between 2 and 5 years with LS were included in the study: three patients (P1–P3) were confirmed to have CIV deficiency based on muscle biopsy and routine spectrophotometric methods, and two new patients (P4 and P5) were clinical suspects for CIV deficiency. All children were born after uncomplicated pregnancy and early postnatal adaptation was uneventful. The age at the onset of the disease based on clinical and laboratory data are shown in Tables 1 and 2. Hypotonia, failure to thrive, growth retardation, hypertrichosis and progressive delay and regress of psychomotor functions developed in all patients. Most of the children exhibited progressive cerebellar symptomatology and attacks of hyperventilation during metabolic acidosis events. Mild to moderate hypertrophy of the interventricular septum, usually non-progressive, was frequently found upon echocardiographic examination.

Spectrophotometric measurements of the catalytic activity of OXPHOS complexes in muscle mitochondria and in cultivated fibroblasts of P1–P3 revealed CIV deficiency, and the activities of other respiratory complexes were in normal range. In all three patients, the catalytic activities of CIV in isolated muscle mitochondria and in cultivated fibroblasts were lower than in controls by 8–12% and 24–37%, respectively. The amount of CIV protein was also lower. The results of 2D BN-PAGE obtained for P1 are shown in Fig. 1. DNA analyses of samples obtained from P1–P3 revealed the presence of pathologic mutations in the SURF1 gene (Table 2).

The control group comprised five healthy children of similar age to the test group, and sample stability was tested in a group of five adult controls. Informed consent was obtained prior to taking samples, and the study was approved by the Ethical Committee of the General University Hospital in Prague.

**Buccal epithelial cells**

Buccal epithelial cells were collected using a Sterile OmniSwab (Cat. No. WB100035, Whatman, Whatman/GE Healthcare Europe GmbH, Freiburg, Germany). The

Table 1. Age at onset and symptoms in five children with Leigh syndrome and cytochrome c oxidase deficiency

<table>
<thead>
<tr>
<th>Case</th>
<th>Age at onset (months)</th>
<th>First symptoms</th>
<th>Developmental decay</th>
<th>Failure to thrive / short stature</th>
<th>Neurological symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>10</td>
<td>hypotonia, failure to thrive</td>
<td>++</td>
<td>++ / ++</td>
<td>cerebellar symptoms, tremor</td>
</tr>
<tr>
<td>P2</td>
<td>6</td>
<td>hypotonia</td>
<td>++</td>
<td>++ / ++</td>
<td>quadruparesis, myoclony, tremor</td>
</tr>
<tr>
<td>P3</td>
<td>18</td>
<td>hypotonia</td>
<td>++</td>
<td>++ / ++</td>
<td>cerebellar symptoms, tremor, ptosis, PEO*</td>
</tr>
<tr>
<td>P4</td>
<td>10</td>
<td>hypotonia, failure to thrive</td>
<td>++</td>
<td>++ / ++</td>
<td>paleocerebellar symptoms</td>
</tr>
<tr>
<td>P5</td>
<td>3</td>
<td>hypotonia</td>
<td>++</td>
<td>++ / ++</td>
<td>cerebellar symptoms</td>
</tr>
</tbody>
</table>

*PEO – progressive external ophthalmoplegia

Table 2. Laboratory findings in five children with Leigh syndrome and cytochrome c oxidase deficiency

<table>
<thead>
<tr>
<th>Case</th>
<th>B-lactate (mmol/l)</th>
<th>Metabolic acidosis events</th>
<th>Echocardiography</th>
<th>Electromyography</th>
<th>Mutation in the SURF1 gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>2.00-11.00 CSF 4.2</td>
<td>++</td>
<td>normal</td>
<td>polyneuropathy</td>
<td>c.312_321del10insAT</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>c.845_846delCT</td>
</tr>
<tr>
<td>P2</td>
<td>2.70-3.09</td>
<td>++</td>
<td>non-progressive IVS* hypertrophy (187%)</td>
<td>nd**</td>
<td>c.845_846delCT</td>
</tr>
<tr>
<td>P3</td>
<td>1.30-7.50 CSF 3.6</td>
<td>++</td>
<td>progressive IVS* hypertrophy (100-204-250%)</td>
<td>polyneuropathy</td>
<td>c.754_755delAG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>c.845_846delCT</td>
</tr>
<tr>
<td>P4</td>
<td>2.60-10.20</td>
<td>++</td>
<td>normal</td>
<td>polyneuropathy</td>
<td>c.845_846delCT</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>c.845_846delCT ***</td>
</tr>
<tr>
<td>P5</td>
<td>3.90-7.80 CSF 5.2</td>
<td>++</td>
<td>normal</td>
<td>nd**</td>
<td>c.312_321del10insAT</td>
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<td></td>
<td></td>
<td></td>
<td>c.312_321del10insAT***</td>
</tr>
</tbody>
</table>

*IVS – interventricular septum, **nd – not done, *** – molecular analysis of P4 and P5 was performed during this study to confirm mutations in the SURF1 gene
cotton brush on the OmniSwab was used to scrape the inside of both cheeks. The cheeks were scraped gently to prevent injury and blood contamination of the buccal epithelial cells. The cotton brush containing the collected cells was transferred to a clean microtube and stored at -80 °C until use.

To test sample stability, 5 × 2 samples of buccal swabs from each control were obtained at intervals. The first swab was frozen within 20 min, and later swabs were frozen at 2-hour intervals (2, 4, 6 and 8 h); all swabs were stored at -80 °C.

Fibroblasts

Fibroblasts of patients P1–P3 and two age-related controls were cultivated in Quantum 333 Medium for Fibroblasts containing L-glutamine (U15-813, PAA Laboratories GmbH, Pasching, Austria) at 37 °C under 5% CO₂ atmosphere. Pellets of harvested fibroblasts were prepared from each T75 cm² cultivating bottle from each patient and from the control; the pellets were then stored at -80 °C.

Dipstick immunocapture analysis

Two DIA kits (ab109722, ab109877, Complex I and IV Human Protein Quantity Dipstick Assay Kit, Mitosciences) were used to determine the levels of CI and CIV in the buccal epithelial cells and fibroblasts. The experiments were carried out according to the manufacturer’s instructions with minor modifications, as described briefly below.

Buccal cell isolation

Buccal cell isolation was performed by adding 600 µl of buffer A (ab109722 or ab109877) and 1% protease inhibitory cocktail (PIC, Sigma-Aldrich, St. Louis, MO) to an Eppendorf tube (Eppendorf, Hamburg, Germany) containing a swab. The tube was incubated on ice with frequent mixing for one hour and then centrifuged at 15 000 g for 20 min at 4 °C. The supernatant (cell lysate) was used for further analyses or stored at -80 °C.

Cell pellets of fibroblasts

Cell pellets of fibroblasts obtained from patients P1–P3 and the controls were treated in the same way as described above, except that the cells were incubated for 20 min in 300 µl of buffer A containing 1% PIC (Sigma-Aldrich).

Calibration curves

In the first instance, calibration curves were prepared to determine the ideal size of buccal swab samples for use in determining the levels of respiratory chain CI and CIV proteins using DIA. Protein concentrations from 50 to 150 µg per assay were tested.

Calibration curves were prepared for use in determining the quantity and activity of CI and CIV in fibroblasts; 5 to 50 µg of protein was tested per assay (data not shown).

Dipstick analysis to determine the amounts of complex I and complex IV in buccal epithelial cells and fibroblasts

One hundred micrograms of protein per buccal cell lysate sample was diluted to 50 µl, and 20 µg of fibroblast lysate sample was diluted to 25 µl in Buffer A. Buffer B (25 µl) was then added to the sample, and the mixture was transferred to a microplate well containing dried gold-conjugated antibody (ab109722 or ab109877). After antibody rehydration, the dipstick was added to the microplate well, and the sample was allowed to wick onto it. Then, 30 µl of washing buffer (Buffer C) was added into the well and allowed to wick onto the dipstick to improve the contrast. After drying, the dipstick was scanned, and the signal intensity of CIV/CI was
measured using Quantity One 1-D Analysis Software (BIO-RAD Laboratories, Hercules, CA).

**Dipstick analysis of complex I and complex IV activity in fibroblasts**

The Complex I Enzyme Activity Dipstick Assay Kit and the Complex IV Human Enzyme Activity Dipstick Assay Kit were used to measure the CI and CIV activity. CI and CIV activity assay procedures are similar to those used in quantitative kits, and the experiments were carried out according to the manufacturer’s protocol. In each analysis, 25 µg of protein (CI activity assay, ab109720, MitoSciences) or 45 µg of protein (CIV activity assay, ab109876, MitoSciences) was used to determine the catalytic activity. The signal intensity representing CIV/CI activity was processed using Quantity One 1-D Analysis Software (BIO-RAD Laboratories). Protein concentration was measured in all samples using Folin’s phenol reagent and the Lowry method (Lowry et al., 1951).

**2-Dimensional blue native polyacrylamide gel electrophoresis**

2-Dimensional blue native polyacrylamide gel electrophoresis (2D BN-PAGE) was used for separation of mitochondrial OXPHOS complexes in polyacrylamide 6–15% (w/v) gradient gel using a MiniProtean® 3 System (BIO-RAD Laboratories). Isolated muscle mitochondria were solubilized with DDM (n-dodecyl β-D-maltoside; Sigma-Aldrich) with a final DDM/protein ratio of 1.0 mg/mg in a buffer containing 1.5 M aminocaproic acid, 2 mM EDTA and 50 mM Bis-Tris (pH 7.0) at 4 °C. Serva Blue G (Serva Electrophoresis GmbH, Heidelberg, Germany) was added to the solubilized protein at a concentration of 0.1 mg/mg of detergent and 10 µg of protein was loaded for each lane. The electrophoresis was performed at 40 V, 4 °C for 1 h and then at 100 V, 4 °C.

For the second dimension, strips of the first-dimension gels were incubated for 40 min in 1% 2-mercaptoethanol and 1% SDS and denatured proteins were then resolved in the second dimension of 12% polyacrylamide 0.1% SDS gel (Schagger and von Jagow, 1991). Subunits of OXPHOS complexes from the second dimension were visualized using silver staining.

**DNA analysis and sequencing**

DNA was isolated from leukocytes according to standard purification protocols (QIAamp DNA mini kit, Qiagen kit or phenol extraction, Hilden, Germany). All nine exons of the SURF1 gene in five fragments were analysed by cycle sequencing using universal primers. The sequenced fragments were amplified using PCR and purified by gel extraction.

**Statistics**

The results of three independent measurements conducted under the same conditions were evaluated separately using Quantity One 1-D Analysis Software (BIO-RAD Laboratories), and the resulting scores were averaged and interpreted.

**Results**

**Calibration curves**

First, calibration curves were prepared to determine the appropriate amount of buccal swab samples for determining the amounts of respiratory chain CI and CIV present using DIA. Protein concentrations from 50 to 150 µg per assay were tested, and 100 µg was found to provide acceptable results for both the CI and CIV (Fig. 2).

**Sample stability test**

The stability of epithelial cells was tested in the buccal swab samples from five adult controls by altering the delay between material withdrawal and freezing (20 min and 2, 4, 6 and 8 h). All samples were analysed

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**Fig. 2.** Calibration curves used for the measurement of the amounts of respiratory chain complexes IV (A) and I (B) in buccal epithelial cells. Protein concentration was determined using the Lowry method, and the analyses were performed using DIA (ab109877, ab109722, MitoSciences). The signal intensity was quantified using Quantity One 1-D Analysis Software (BIO-RAD Laboratories). Each point corresponds to the average value of two independent measurements of adult control. The amount of applied protein followed the MitoSciences protocol recommendation. Repetition with other control samples showed similar calibration curves.
using DIA (ab109722, ab109877, MitoSciences), and the signal intensities were compared between different delay times (Fig. 3). Our results showed that the samples that were frozen later provided lower signal intensity than samples that were frozen earlier. CI and CIV exhibited similar negative trends (Fig. 3). To prevent obtaining false-positive results, we decided to use only buccal smears that were frozen less than 4 h after collection.

**Patient testing**

DIA (ab109722, ab109877, MitoSciences) was used to determine the amounts of CI and CIV in buccal epithelial cells obtained from five patients and five age-related controls. The results of three independent measurements obtained under the same conditions were evaluated separately using Quantity One 1-D Analysis Software (BIO-RAD Laboratories), and the scores were averaged and interpreted.

Lower levels of CIV were found in buccal epithelial cells in all three patients in which CIV deficiency had been confirmed using muscle biopsy (P1 69.6 %, P2 64.7 %, P3 65.5 %) and the two new patients, P4 and 5 (P4 62.6 %, P5 86.8 %), than in the controls (Fig. 4A).

The level of CI was variable in buccal epithelial cells; the level of CI was lower (83 %) in P4, unchanged in P1 (104 %), P2 (118 %) and P3 (98 %), and higher in P5 (132 %) than in controls of similar age (Fig. 4B).

DIA was used to determine the amount and activity of CI and CIV in fibroblasts obtained from patients P1–P3 (Fig. 5A, B, C, D). A critical decrease in the amount of CIV was detected in the fibroblasts of all patients, and the signal obtained using the dipsticks was almost undetectable in the patient samples. The values found in patients were less than 1 % of those found in the controls. The catalytic activity of CIV found in fibroblasts was less than 20 % of that of the controls in all three patients (P1 11 %, P2 19.6 % and P3 15 %). The amounts and activities of CI were also lower in fibroblasts obtained from patients P1–P3 than in the controls. The amount of CI in patient fibroblasts was between 5 % and 37 % of that of the controls. The catalytic activity of CI decreased similarly to the amount. The activities of CI in patients P1–P3 were 13, 53 and 68 % of the control values, respectively.

Molecular analysis of the SURF1 gene confirmed that P4 is homozygous for c.845_846delCT, and P5 is homozygous for the mutation c.312_321del10insAT.

**Discussion**

Mitochondrial diseases represent a heterogeneous group of disorders with a total incidence of approximately 1 : 5000 (Thorburn, 2004). More than 220 pathological mutations in mitochondrial and nuclear genes that participate in the assembly and function of OXPHOS have been detected (Distelmaier et al., 2009; Koenig, 2008). Disturbances of CI and CIV represent the most common group of mitochondrial defects in men (Robinson, 2000). The diagnosis of mitochondrial disorders is difficult in many patients due to overlapping symptoms and tissue-specific changes that often result in the need for invasive procedures, including muscle biopsies.
New biochemical methods for use with non-invasively obtained samples, including cells obtained from a buccal smear, may be of great importance for some patients with respiratory chain CI and CIV deficiencies (Goldenthal et al., 2011). In our study of children with LS, the use of DIA for the determination of CI and CIV in buccal cells confirmed that this technique presents advantages for diagnosis. It appears that non-invasively obtained tissues such as buccal epithelial cells may be used in screening for mitochondrial disorders. We have shown that 100 µg of protein obtained from buccal epithelial cells is suitable for diagnosis. In addition, it is important that the samples should be frozen shortly after collection due to the risk of protein degradation; this is true for both complexes, especially for CIV, which seems to be more sensitive (Fig. 3).

Lower amounts of CIV were detected in buccal cells in all three patients with LS for whom CIV deficiency had been confirmed using muscle biopsy. Molecular analyses revealed that all of these patients are homozygous or compound heterozygous for pathological mutations in the SURF1 gene. Impaired function of the Surf1 protein, an essential factor for the early assembly stages of CIV, is associated with lower amounts of CIV because Surf1 deficiency results in reduced levels and instability of fully assembled and functional CIV (Tiranti et al., 1998). In the remaining two children, DIA analyses demonstrated low amounts of CIV in buccal cells, enabling us to cancel the originally indicated muscle biopsies and to continue directly with molecular analyses. Subsequently, pathological mutations in the SURF1 gene were found in both children.

Decreased amounts of CIV in the buccal epithelial cells of patients P1–P3 correlated not only with the results of biochemical analyses of muscle biopsy samples, but also with decreased amounts of CIV in cultivated fibroblasts as analysed using spectrophotometrical methods (data not shown). In patients P1–P3, we also performed DIA analyses to determine the amount and activity of CIV in cultivated fibroblasts and found that...
both were significantly lower in all patients than in the controls of similar age (Fig. 5A, B). In addition, the amount of CI was also lower in cultivated fibroblasts than in the controls (Fig. 5C, D). This decrease might be explained by arrangements of supercomplexes containing CI, CIII and CIV. When the level of CIV is decreased, the supercomplexes might not form correctly, and the stability and function of CI would be disrupted (Diaz et al., 2006). Contrary to the results obtained using DIA for the fibroblast samples, the amount of CI in buccal epithelial cells was lower in one patient only. We propose that the different results obtained using fibroblasts and buccal cells in our study might be caused by the tissue-specific manifestation of the disease and/or different effects of other OXPHOS complexes that compensate for CIV deficiency in our patients (Stiburek et al., 2005). This compensation theory is supported by an analysis of OXPHOS complexes that calculated from cultivated fibroblasts obtained from nine patients with mutations in SURF1 (Kovářová et al., 2012), where 32–54 % up-regulation of CI, CIII and CV and accumulation of Cox5a subunit was found.

In our study, we focused especially on analysing the amount of CIV, because most mutations in the SURF1 gene are loss-of-function mutations and changes in the amount of CIV are expected.

The use of the DIA method was sensitive enough to detect CIV deficiency in buccal epithelial cells in all tested patients, who exhibited various mutations in the SURF1 gene. The DIA method is strongly advantageous in that it utilizes cells obtained from the buccal epithelium, which can be obtained non-invasively and repeatedly. This aspect is of importance for use with small children, especially in patients with mutations in the SURF1 gene. The method is very rapid; sample preparation, protein concentration measurement, dipstick analysis and evaluation of the results can be concluded in approximately 7 h. Thus, the results are available within one day. Buccal smears and cell lysates can be subjected to long-term storage at -80 °C. Additionally, the process does not demand expensive equipment. Moreover, this method uses small amounts of material and is not limited to buccal smears. DIA was successfully tested in our laboratory not only using cultivated fibroblasts, but also using isolated lymphocytes and platelets (data not shown).

Conclusions: The dipstick immunocapture assay (DIA) is a simple, rapid and sensitive method for non-invasive screening and diagnosis in patients with mitochondrial disorders resulting from CIV deficiency. In addition, the method may aid in the differential diagnosis of other metabolic diseases.

References