Mutation Analysis of the Pyruvate Dehydrogenase 
$E_1\alpha$ Gene in Eight Patients With a Pyruvate 
Dehydrogenase Complex Deficiency

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Most of the mutations causing deficiency of the pyruvate dehydrogenase (PDH) complex are in the 
X-linked $E_1\alpha$ gene. We have developed a rapid screening method for the detection of mutations in this 
gene using reverse transcription of total RNA, polymerase chain reaction amplification of the whole 
coding region of the gene and single-strand conformation polymorphism (SSCP) analysis. With this 
method, we studied eight patients with a PDH complex deficiency, using cultured fibroblasts. In all 
patients, aberrant SSCP patterns were found and, after sequencing of the corresponding fragments, we 
were able to identify six new mutations and two mutations already described previously. The mutations 
are point mutations leading to amino acid substitutions (5) and direct repeat insertions (3). The 
presence of the mutations was confirmed in genomic fibroblast DNA. The 4 female patients were 
shown to carry both a normal and a mutated $E_1\alpha$ gene. © 1996 Wiley-Liss, Inc.

KEY WORDS: Pyruvate dehydrogenase $E_1\alpha$, RT-PCR, SSCP, Mutation analysis

INTRODUCTION

The pyruvate dehydrogenase (PDH) complex catalyzes the irreversible conversion of pyruvate to 
acetyl-CoA. The complex is localized in the mitochondria and connects glycolysis with the citric 
acid cycle and oxidative phosphorylation. It consists of multiple copies of three catalytic enzymes 
$E_1$, $E_2$, and $E_3$, a protein with an unknown function (protein X) and two enzymes regulating the 
activation/inactivation of the $E_1$ enzyme.

Human PDH complex deficiency is one of the most prominent causes of primary congenital lactic 
acidosis (Robinson, 1989, 1993). Studies at both the molecular and protein levels have demonstrated that in most patients the reduction in activity of the complex is due to defects in the $E_1$ component (Robinson, 1989; Dahl et al., 1992a).
The $E_1$ enzyme is a heterotetramer of $2\alpha$ and $2\beta$-subunits, but so far only mutations in the $E_1\alpha$ gene 
have been found. This gene is approximately 17 kilobase (kb) long, contains 11 exons, and is localized on the X-chromosome in band Xp22.1 (Koike et al., 1990; Maragos et al., 1989; Brown et al., 1989). mRNA is expressed in all tissues and is approximately 1.5 kb long, encoding a protein of 390 amino acids (Dahl et al., 1987; De Meirleir et al., 1988; Koike et al., 1988; Ho et al., 1989). The diagnosis of PDH complex deficiency is based primarily on the measurement of lowered enzyme complex activity in tissues of the patients. Cultured skin fibroblasts are most frequently used for diagnosis. As previously mentioned, most of the mutations in PDH complex deficiencies are found in the $E_1\alpha$-subunit of the complex; however,

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the expression of this gene at the mRNA level does not appear to be altered in most of the patients (Dahl et al., 1992a,b; Dahl and Brown, 1994; De Meirleir et al., 1992, 1993, 1994; Chun et al., 1993; Wexler et al., 1992; Matthews et al., 1993; Hansen et al., 1993; Takakubo et al., 1993a,b; Endo et al., 1989, 1991). We describe the detection of mutations in the E1α gene of eight patients with a PDH complex deficiency. Mutation detection was based on reverse transcription-polymerase chain reaction (RT-PCR) of the whole coding region of the E1α gene, starting from mRNA isolated from fibroblasts, followed by single-strand conformation polymorphism (SSCP) analysis (Orita et al., 1989) and direct sequencing of aberrant SSCP fragments.

MATERIALS AND METHODS
RNA Preparation and Reverse Transcription

Fibroblasts of the patients were grown in Opti­mem I medium (Gibco, Ghent, Belgium), supple­mented with 5% fetal calf serum (FCS), 2 mM glutamine, and 50 IU/ml penicillin/streptomycin. Total RNA was prepared from one or two 250-ml tissue culture flasks (Gibco) according to the method of Chomczynski and Sacchi (1987). Five μg of total RNA was used for first-strand cDNA synthesis (Frist-strand cDNA synthesis kit, Pharmacia, Roosendaal, the Netherlands), according to the manufacturer's instructions in a total volume of 33 μl.

PCR and SSCP Analysis of the PDH E1α Gene

The whole coding sequence of the PDH E1α gene was PCR amplified in six consecutive overlapping fragments (Table 1, fragments A–F). All reactions were performed in a final volume of 50 μl containing 5 μl of first-strand cDNA mixture, 10 pmol of each primer, 50 mM KCl, 10 mM Tris–HCl pH 8.3, 2 mM MgCl₂, 2 units of Taq poly­merase (Perkin-Elmer Cetus, Norwalk, CT), 10 μCi of 35S-dATP and 35S-dCTP (1,000 Ci/mmol; Amersham, England) and 0.01% gelatin. The addition of nonradioactive nucleotides is unnecessary due to carryover from the first-strand cDNA mixture. Thermal cycling conditions were denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 45 sec, for 30 cycles, and a final extension at 72°C for 7 min. For SSCP analysis, 2.5 μl of each PCR product was mixed with 10 μl of a solution containing 0.1% sodium dodecyl sulfate (SDS) and 10 mM EDTA, pH 8.0, and 10 μl of 95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol FF, and 20 mM EDTA, pH 8.0. Five μl was removed for the identification of double-stranded fragments; the remainder was heated to 85°C for 5 min and then immediately placed on ice. Three μl of nondenatured and de­natured samples was then loaded on either 6% or 8% polyacrylamide SSCP gels (acrylamide-to­bisacrylamide ratio 29:1 and 37.5:1) with or with­out 5% glycerol. The different conditions of elec­trohoresis described by Michaud et al. (1992) were used. After electrophoresis, the gels were fixed in 10% of acetic acid/10% of methanol and dried on filter paper. Autoradiography was performed for 24–48 hr.

DNA Sequencing of PDH E1α cDNA Fragments

After reverse transcription, the 6 PDH E1α cDNA fragments were PCR amplified in a total volume of 100 μl. The reaction conditions were essentially the same as described above for SSCP analysis, except that 200 μM each of dNTP and 100 pmol of each primer was added. Radioactive nucleotides were omitted from the PCR mixtures,
TABLE 2. Summary of PDH E1α Mutations

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>PDH complex activity (%)</th>
<th>SSCP fragment</th>
<th>Nucleotide change</th>
<th>Exon</th>
<th>Amino acid change</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>30</td>
<td>A</td>
<td>C→T at 319</td>
<td>3</td>
<td>R72C</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>10</td>
<td>E</td>
<td>C→T at 1008</td>
<td>10</td>
<td>R302C</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>12</td>
<td>B</td>
<td>C→G at 442</td>
<td>4</td>
<td>H113D</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>40</td>
<td>B</td>
<td>G→A at 589</td>
<td>5</td>
<td>G162R</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>31</td>
<td>D/E</td>
<td>C→G at 892</td>
<td>8</td>
<td>R263G</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>15</td>
<td>F</td>
<td>1,264ins39⁴</td>
<td>11</td>
<td>K387fs</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>29</td>
<td>F</td>
<td>1,217ins13⁴</td>
<td>11</td>
<td>S371fs</td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>15</td>
<td>E/F</td>
<td>1,069ins15⁴</td>
<td>10</td>
<td>D322fs</td>
</tr>
</tbody>
</table>

*Sex: M, male; F, female.*

*Activities (in fibroblasts) are expressed as percentage of control mean. Mean 1.62 nmol/mg/min; range 1.21–2.81 nmol/mg/min (De Meirleir et al., 1992).*

*Direct repeat of bases 1,226–1,264 after 1,264.*

*Bases 1,204–1,216 after 1,217.*

*Bases 1,055–1,069 after 1,069.*

and an extension time of 1 min was used. After amplification, the PCR products were purified on a Centricon 100 microconcentrator (Amicon, Beverly, MA, USA) and sequenced in both directions by dideoxynucleotide chain termination with the PCR primers, using Sequenase Version 2.0 kit (US Biochemical Corp., Beverly, MA, USA).

**Patients**

All patients studied had PDH complex activity in fibroblasts, 10–50% of the mean of our control values (Table 2). Patient 1 is a boy of nonconsanguinous parents. His initial development was normal. At the age of 2.5 years, during an intercurrent infection, he became severely ataxic and hypotonic, but he recovered within a few days. At the age of 3 years, he presented with easy fatigability and difficulties in climbing the stairs. This was followed by episodes of dystonia of the lower limbs. Ataxia and weakness increased progressively, preventing independent walking. Metabolic workup revealed an increased blood lactate and pyruvate, with normal lactate/pyruvate ratio. A sensorimotor neuropathy was found. Magnetic resonance imaging (MRI) of the brain showed hypodensities in basal ganglia. PDH activity in fibroblasts was 30% of normal. His condition continues to progress.

The clinical picture of patient 2 was very similar to that of a patient described previously (De Meirleir et al., 1993). She presented with a severe developmental delay since birth. Myoclonic seizures started at the age of 4 months. She also had agenesis of the corpus callosum, as well as cortical atrophy and microcephaly. PDH activity measured in fibroblasts was 30% of normal. Her condition continues to progress.

Patient 3 has been described previously (De Meirleir et al., 1992). She presented with severe ataxia and hypotonia, but recovered within a few days. At the age of 3 years, she presented with easy fatigability and difficulties in climbing the stairs. This was followed by episodes of dystonia of the lower limbs. Ataxia and weakness increased progressively, preventing independent walking. Metabolic workup revealed an increased blood lactate and pyruvate, with normal lactate/pyruvate ratio. A sensorimotor neuropathy was found. Magnetic resonance imaging (MRI) of the brain showed hypodensities in basal ganglia. PDH activity in fibroblasts was 30% of normal. His condition continues to progress.

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In patient 3, a developmental delay with hypotonia was noted at the age of seven months. She developed spastic diplegia and microcephaly. At the age of seven years, she was severely retarded. PDH activity in fibroblasts was 12% of normal.

Patient 4 also presented with developmental delay and microcephaly. Spastic quadriplegia developed, and she became severely retarded. Computed tomography (CT) scan of the brain demonstrated severe cortical atrophy, and ventricular dilation was seen. PDH activity in fibroblasts was 40% of normal.

Patient 5 is a boy who was admitted at the age of 16 months with an episode of somnolence, hyperventilation and choreic movements. Four maternal brothers died in the neonatal period of unknown cause. Blood lactate and pyruvate were elevated. MRI of the brain showed hyperintensities in the caudal and lenticular nucleus. A diagnosis of Leigh's encephalomyelopathy was proposed. PDH activity in fibroblasts was 31% of normal.

Patient 6 is a boy presenting with severe lactic acidosis. He died at the age of 18 months. No further clinical details are available. PDH activity in fibroblasts was 15% of normal.

Patient 7 has been previously described (Sengers et al., 1983). Patient 8 is a boy presenting with early onset lactic acidosis, hyperventilation, developmental delay, and failure to thrive. He died at the age of 15 months. PDH activity in fibroblasts was 15% of normal.

**RESULTS**

To permit rapid screening for mutations in the PDH E1α gene, we have designed 12 oligonucleotide primers that permit amplification by PCR of the whole coding sequence of the gene in six consecutive overlapping fragments (Table 1). After reverse transcription of total RNA, isolated from fibroblasts from eight patients and PCR amplifica-
The abnormal SSCP analysis. For all eight patients, abnormal migrating cDNAs, the fragments were subjected to SSCP analysis of all female patients was confirmed. The presence sequenced in both directions. In each case, a mutation of the six fragments starting from the resulting fragments were systematically less intense than the aberrant (mutant) fragments.

The corresponding fragments were re-amplified from cDNA from each of these eight patients and sequenced in both directions. In each case, a mutation was found (Table 2). The sequencing results for patients 4, 6, 7, and 8 were reviewed by the editors, but are not shown. The heterozygote status of all female patients was confirmed. The presence of these mutations was confirmed in genomic fibroblast DNA of each patient. As expected, for the two patients showing two abnormal SSCP fragments (patients 5 and 8), the mutation was found in the overlap region of the SSCP fragments.

DISCUSSION

Deficiency of the PDH complex is mostly due to defects in the E1 component of the enzyme (Robinson, 1991). So far, no mutations have been found in the β-subunit of E1 (Chun et al., 1993), but about 30 mutations have been identified in the α-subunit. We have developed a rapid screening method for the detection of mutations in the E1α gene in patients with a PDH complex deficiency. It is based on RT-PCR, followed by SSCP analysis and direct sequencing of aberrant SSCP fragments. We have used this screening method for the analysis of the E1α gene in eight patients; aberrant SSCP fragments were found for each patient. Direct sequencing of the corresponding fragments revealed missense mutations in five patients and direct repeat insertions leading to a shift in the amino acid reading frame of the E1α gene in the three other patients. The presence of the mutations was confirmed in genomic DNA of all patients. The four female patients analyzed in this study were, as expected, carriers of both a normal and a mutated E1α gene. They predominantly express the mutated gene through nonrandom X-inactivation of the X-chromosome containing the normal E1α gene. This is supported by the low residual PDH complex activity (Table 1) and is also seen in SSCP analysis where the normal SSCP fragments were systematically less intense than the aberrant (mutant) fragments.

The five missense mutations are located in exons 3 (R72C), 4 (H113D), 5 (G162R), 8 (R263G), and 10 (R302C) and all involve a considerable change in the charge of the predicted amino acid substitution. Four of these mutations are in a CpG dinucleotide (R72C, G162R, R263G, and R302C), and three of them (R72C, G162R and R302C) could be explained by spontaneous deamination of 5-methylcytosine (Cooper and Krawczak, 1993). The amino acids at positions 72, 113, 162, 263, and 302 of the human E1α gene are all conserved in the somatic E1α genes of rat, mouse, and pig (Matuda et al., 1991; Fitzgerald et al., 1992; Sermon et al., 1990) and in the testis-specific E1α genes of human and mouse (Dahl et al., 1990; Fitzgerald et al., 1992). With the exception of the mother of patient 5, these mutations were also absent in the genomic DNA from leukocytes of the parents of the patients. Therefore, we believe that these mutations are disease causing. In patients 6, 7, and 8, the insertions introduce a shift in the amino acid reading frame. The direct repeat insertions in these patients predict truncated proteins starting from codons 387, 372, and 322, respectively, that are 13, 32, and 5 amino acids longer than native E1α. The mutation in patient 8 was not present in genomic leukocyte DNA of his mother, indicating that the mutation either occurred de novo or is present in the germ-line of the mother. The parents of the two other patients were not available for study.

Two of the eight mutations found in our patients (R263G and R302C) have already been described previously (Wexler et al., 1992; Chun et al., 1993; Dahl et al., 1992b; De Meirleir et al., 1993). The clinical history of female patient 2 (R302C mutation) is very similar to already described patients carrying this mutation. This is also true for patient 7 with a 13-bp repeat insertion in exon 11 (Sengers et al., 1983). These girls presented with symptoms in the neonatal period and have severe developmental delay, dysmorphism, bouts of lactic acidosis, seizures and microcephaly with dilated ventricles. By contrast, in female patients 3 and 4, who have mutations in exons 4 and 5, the clinical onset is delayed until a few months after birth. They do not have severe cortical atrophy or developmental brain anomalies as in the two other females, but the clinical picture again results in a severe mental retardation with microcephaly and spastic quadriplegia.

The mutation R263G has previously been asso-
associated with Leigh's encephalomyelopathy. The clinical picture of patient 5 is in accordance with this. The patient carrying the R72C mutation, patient 1, also fits this picture with episodic ataxia associated with muscle weakness as an initial symptom and progressing into a Leigh's encephalomyelopathy. The clinical normal mother of patient 5 is a carrier of a normal and a mutated R263G E\(\alpha\) gene. In general, mutations in E\(\alpha\) are not present in the parents, suggesting that most PDH mutations represent new mutations (Dahl et al., 1992). The R263G mutation, as well as the R302C mutation, seem to be exceptions. Recently, familial transmission of a mutation leading to partial skipping of exon 6 of the E\(\alpha\) gene has also been demonstrated (De Meirleir et al., 1994). Nonrandom X-inactivation resulting in disproportional expression favoring the normal or the mutated allele could then explain either the normal or the affected phenotype in females carrying one of these mutations. Obviously males carrying the mutated allele would be affected. It has been suggested that the R263G mutation disrupts the subunit-subunit interaction of the E\(\alpha\)- and E\(\beta\)-subunits resulting in the instability of E\(\alpha\). The mother of our patient had four brothers who died in the neonatal period of an unknown cause. Although it has not been possible to study these maternal brothers, the familial history is compatible with a R263G PDH E\(\alpha\) deficiency. The two other boys have mutations in exons 11 and 10 (patients 6 and 8); both presented with a severe neonatal lactic acidosis and early death.

Several methods have been described for the rapid detection of minor changes in nucleic acids (for review, see Grompe, 1993). We have chosen to use SSCP analysis for the detection of mutations in the PDH E\(\alpha\) gene because of its simplicity and sensitivity (Hayashi and Yandell, 1993). All the mutations described in this study were detected in SSCP by using 8% polyacrylamide gels (acylamide-to-bisacrylamide ratio 37.5:1) containing 5% glycerol, at room temperature. Moreover, the patterns obtained from normal control samples were fully reproducible from experiment to experiment. Therefore, we always run the first SSCP under these conditions and only, when no aberrant migrating bands are found in patients, other SSCP conditions are used (Michaud et al., 1992). The possibility of splicing out of an exon from the gene was also largely taken into account by designing the SSCP primers. Splicing out of all exons, except exons 3 and 10, would immediately be obvious from the SSCP patterns. For mutations resulting in splicing out of exons 3 and 10, additional amplifications with primers PDS1/PDS8 and PDS3/PDS6 would be necessary.

The study of X-inactivation patterns, by analyzing the methylation status of the hypervariable DXS255 locus, has been suggested as an aid in the diagnosis in females with a suspected PDH E\(\alpha\) deficiency (Brown and Brown, 1993). However, the methylation status at this locus may be affected by factors other than X-chromosome inactivation (Hendriks et al., 1991; Cachia et al., 1992). Moreover, skewed X-inactivation will only give an indirect indication of a possible involvement of the E\(\alpha\) gene in disease. Since the SSCP system described here permits a direct search for mutations in the gene, we believe that this system should be preferentially used for mutation detection.

In summary, we have developed a RT-PCR-based SSCP assay for the detection of mutations in the PDH E\(\alpha\) gene. Starting from total RNA, the result of the screening can be obtained in 72 hr. We have used this method in eight patients with a PDH complex deficiency and have found mutations in the E\(\alpha\) genes of all patients.

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