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Research paper

Complex genetic findings in a female patient with pyruvate dehydrogenase complex deficiency: Null mutations in the *PDHX* gene associated with unusual expression of the testis-specific *PDHA2* gene in her somatic cells

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ABSTRACT

Human pyruvate dehydrogenase complex (PDC) catalyzes a key step in the generation of cellular energy and is composed by three catalytic elements (E1, E2, E3), one structural subunit (E3-binding protein), and specific regulatory elements, phosphatases and kinases (PDKs, PDPs). The E1α subunit exists as two isoforms encoded by different genes: *PDHA1* located on Xp22.1 and expressed in somatic tissues, and the intronless *PDHA2* located on chromosome 4 and only detected in human spermatocytes and spermatids.

We report on a young adult female patient who has PDC deficiency associated with a compound heterozygosity in *PDHX* encoding the E3-binding protein. Additionally, in the patient and in all members of her immediate family, a full-length testis-specific *PDHA2* mRNA and a 5′UTR-truncated *PDHA1* mRNA were detected in circulating lymphocytes and cultured fibroblasts, being both mRNAs translated into full-length PDHA2 and PDHA1 proteins, resulting in the co-existence of both PDHA isoforms in somatic cells. Moreover, we observed that DNA hypomethylation of a CpG island in the coding region of *PDHA2* gene is associated with the somatic activation of this gene transcription in these individuals.

This study represents the first natural model of the de-repression of the testis-specific *PDHA2* gene in human somatic cells, and raises some questions related to the somatic activation of this gene as a potential therapeutic approach for most forms of PDC deficiency.

1. Introduction

Pyruvate dehydrogenase complex (PDC) catalyzes the irreversible decarboxylation of pyruvate into acetyl-CoA, with concomitant formation of NADH. All subunits are encoded by nuclear genes and imported into mitochondria after cleavage of their leader sequences.

The human complex is formed by multiple copies of three catalytic, one binding and two regulatory components: pyruvate dehydrogenase (E1), dihydrolipoamide acetyltransferase (E2), dihydrolipoyl dehydrogenase (E3), a structural protein (E3-binding protein or Protein X), pyruvate dehydrogenase kinases (PDKs) and pyruvate dehydrogenase phosphatases (PDPs) (Patel et al., 2014). The E1 enzyme (EC 1.2.4.1) is a heterotetramer ($\alpha 2\beta 2$) that catalyzes the decarboxylation of pyruvate by a postulated 'flip-flop' mechanism (Ciszak et al., 2003). The α subunit, besides forming with the β subunit the substrate and the cofactor binding sites, is also the target for the regulatory mechanisms based upon dephosphorylation (activation) and phosphorylation (inactivation) of three serine residues (Patel and Korotchkina, 2001; Harris et al., 2002).

Abbreviations: PDC, pyruvate dehydrogenase complex; SDS-PAGE, sodium dodecyl sulfate - polyacrylamide gel electrophoresis; IEF, isoelectric focusing; IP, isoelectric point; qPCR, quantitative real time PCR; DMR, differentially methylated region; 5'RACE, rapid amplification of cDNA 5' ends.

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PDC deficiency is one of the most common neurodegenerative disorders associated with altered mitochondrial metabolism leading to energy deprivation, especially in the CNS (DeMeirleir, 2013; DeBrosse et al., 2012). Genetic defects of PDC are associated with a variety of neurologic manifestations, ranging from mild ataxia to profound psychomotor retardation and even death during early infancy. Though mutations have already been identified in genes encoding all subunits, the most frequent form of genetically determined PDC deficiency is caused by alterations in the E1α subunit (OMIM #312170), being mostly sporadic and with a very low recurrence rate (Imbard et al., 2011).

The PDC E1α subunit exists as two isoforms encoded by different genes. *PDHA1* is located on region p22.1 of the X chromosome, contains 11 exons, spans approximately 17 kb of genomic DNA and is ubiquitously expressed in somatic tissues. An autosomal *locus*, *PDHA2*, lies on chromosome 4 (4q22–23), completely lacks introns, spans 1.4 kb of genomic DNA, displays characteristics of a functional processed gene and is expressed only in testis after the onset of spermatogenesis (Dahl et al., 1990).

The autosomal localization of *PDHA2* gene is particularly significant in males since X chromosome inactivation occurs early in meiosis and only half of the haploid spermatids contain the X chromosome. During the second meiotic division the spermatids go through a period of maturation, storage and release, in which the haploid sperm, as the brain, is dependent on energy generated from pyruvate *via* the PDC. Therefore, the existence of an autosomal variant of E1α ensures that cellular energy requirements are maintained during spermatogenesis (Fitzgerald et al., 1994; Dahl et al., 2001).

It would be possibly important if *PDHA2* gene could be switched on in somatic tissues of patients with PDC deficiency caused by mutations in *PDHA1* gene (Robinson et al., 1996; Datta et al., 1999), since it could open up new therapeutic avenues for this metabolic disease. It is therefore important to unravel the molecular mechanisms underlying *PDHA2* gene expression in humans. Previous studies reported such mechanisms in the mouse orthologue (Iannello et al., 1993, 1997, 2000), but it is believed that both mouse and human promoter regions evolved from different retroposons (Fitzgerald et al., 1996), because no gross homology exists between the promoters of the mouse and human genes. Accordingly, it was postulated that the regulatory mechanisms underlying the expression of the human *PDHA2* gene should be significantly different from that of rodent species (Datta et al., 1999). Indeed, our group has recently shed some light on this subject, revealing that the transcriptional activity of the human testis-specific *PDHA2* gene correlates with the methylation status of a CpG island located in its open reading frame (Pinheiro et al., 2010), and showing that the tissue-specific expression of *PDHA2* gene is strongly controlled by an epigenetic mechanism, DNA methylation (Pinheiro et al., 2012).

The present report concerns a genetically complex patient carrying a PDC deficiency associated with a compound heterozygosity of two different null mutations in the *PDHX* gene, coding for the E3-binding protein. Additionally, the patient and all members of her immediate family express in circulating lymphocytes and cultured fibroblasts a full-length *PDHA2* mRNA which is translated into PDHA2 protein, as well as a 5′UTR-truncated *PDHA1* mRNA which is also translated into a full-length PDHA1 protein. This is the first reported case where the presence of the *PDHA2* mRNA and protein, until now only detected in human spermatocytes and spermatids, is observed in human somatic cells from a family. These observations raise questions about the potential benefit of somatic activation of the testis-specific *PDHA2* gene expression in other cases of PDC deficiency due to *PDHA1* mutations.

2. Subjects and methods

2.1. Case report

This report was initiated by extensive molecular study of a 27-year-old Portuguese Caucasian girl, first child of non-consanguineous parents, displaying severe psychomotor delay with walking ataxia, encompassing a static encephalopathy. In the neonatal period she developed seizures associated with respiratory distress and up to 3 years of age she was admitted several times due to episodes of lactic acidosis. The patient was referred to our laboratory and her metabolic profile revealed isolated hyperalaninemia (639 μM; normal range 158–314), hyperlactic acidemia (4.4 mM; normal range < 2.2), hyperpyruvic acidemia (0.27 mM; normal range < 0.18) and normal lactate/pyruvate molar ratio (16; normal range < 20). PDC deficiency was confirmed by determination of enzyme activity (Silva et al., 2009) in two different tissues: 21% and 16% of control activity in lymphocytes and fibroblasts, respectively. Her mother also demonstrated reduced enzymatic activity in her lymphocytes and her father showed lower normal activity: 54% and 75% of control activity, respectively [normal values of the laboratory (mean ± SD): 1312 ± 396 pmol/min/mg protein, n = 120]. At 11 years of age, she had been observed at Hôpital Necker-Enfants Malades in Paris, France, where the PDC deficiency was confirmed and attributed most probably to the E1α subunit, since the E1 component displayed 15% of the control activity. Furthermore, cytogenetic analysis performed in Paris revealed a normal karyotype with no skewed X-inactivation. In adulthood, the proband was admitted twice (18 and 21 years of age) with metabolic decompensation induced by lactic acidosis after physical exercise.

This study was approved by the local Ethics Committee and written informed consents were obtained from the parents who were enrolled in the study together with the proband's younger brother.

2.2. Sample preparation

Three independent peripheral blood samples were obtained from the index case and her parents and brother, as well as from control individuals. Lymphocytes were separated at room temperature on a Ficoll-Paque™ gradient (GE Healthcare Bio-Sciences, Uppsala, Sweden).

Patient's fibroblast cultures were established from a diagnostic skin biopsy and grown until confluence in Dulbecco's Modified Eagle Medium (Sigma-Aldrich Inc., St. Louis, MO, USA), supplemented with 10% fetal calf serum (Biochrom AG, Berlin, Germany) and 100 units/mL penicillin (Sigma) and 100 μg/mL streptomycin (Sigma).

Positive controls for *PDHA2* gene expression were obtained from two different sources; a commercially available human testis total RNA sample (Clontech Laboratories Inc., Mountain View, CA, USA) and human testis specimens from eight cases requiring open testicular biopsy for the retrieval of testicular sperm for intracytoplasmic sperm injection. Human sample collection, treatment and preparation have been previously described (Pinheiro et al., 2010).

2.3. Western immunoblotting

Homogenates were prepared from lymphocytes and from whole testis samples using the Cell Lysis Buffer (Cell Signaling Technology, Beverly, MA, USA), which contains protease and phosphatase inhibitors, and directly loaded onto 10% Mini-PROTEAN TGX® Precast™ Gels (Bio-Rad Laboratories Inc., Hercules, CA, USA) and run under reducing conditions. Proteins were electro-transferred onto PVDF membranes (Amersham Hybond™-P, GE Healthcare Bio-Sciences), which were probed with primary antibodies: MitoProfile® Pyruvate dehydrogenase (PDH) WB Antibody Cocktail (MSP07/ab110416, Abcam, Cambridge, UK) and the anti-PDHA1 antibody (MSP03/ab110330, Abcam). Immune-complexes were detected by the LumiGLO® reagent (Cell Signaling Technology).

2.4. Two-dimensional electrophoresis

Homogenates from patient and control lymphocytes were precleared with ReadyPrep™ 2-D kit (Bio-Rad). Pellets were solubilized in 200 μL of a solution containing 8 M urea, 2% (w/v) CHAPS, 50 mM DTT and 1× Bio-Lyte® Ampholyte 20% pH 7–10. An 11 cm, pH 7–10,