

## MUTATION IN BRIEF

# A Novel Mutation in the Dihydrolipoamide Dehydrogenase E3 Subunit Gene (*DLD*) Resulting in an Atypical Form of $\alpha$ -Ketoglutarate Dehydrogenase Deficiency

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The  $\alpha$ -ketoglutarate dehydrogenase complex (KGDC) catalyses the decarboxylation of  $\alpha$ -ketoglutarate into succinyl-coenzyme A in the Krebs cycle. This enzymatic complex is made up of three subunits (E1, encoded by *PDHA1*; E2, encoded by *DLST*; and E3, encoded by *DLD*). The E3 subunit is common to two other enzymatic complexes, namely pyruvate dehydrogenase complex (PDC) and branched-chain ketoacid dehydrogenase complex (BCKDC). KGDC deficiency is a rare autosomal recessive disorder, most often presenting with severe encephalopathy and hyperlactatemia with neonatal onset. We found a KGDC deficiency in cultured skin fibroblasts from three siblings born to consanguineous parents. E3 subunit activity was shown to be deficient (20% of control values), despite the absence of usual clinical clues to E3 deficiency, i.e. accumulation of pyruvate and branched-chain amino acids in plasma and branched-chain  $\alpha$ -ketoacids in urine. RT-PCR of E3 mRNA from the three patients, followed by sequencing, revealed an homozygous c.1444A>G substitution located in E3 exon 13, predictive of a p.R482G (or R447G in the processed gene product) substitution in a highly conserved domain of the protein. Only eleven E3 mutations have been reported so far. The only other case of E3 deficiency without clinical or biochemical evidences of PDC and BCKDC deficiencies has been ascribed to a c.1436A>T (p.D479V; or D444V in the processed gene product) mutation, very close to the mutation reported herein. Since c.1444A>G (p.R482G; or R447G in the processed gene product) and c.1436A>T (p.D479V; or D444V in the processed gene product) lie within the interface domain of E3 with E2 (KGDC and BCKDC) or the E3-binding protein (PDC), our data suggest that interaction of E3 with these other subunits differs in some extent among KGDC, PDC, and BCKDC. © 2005 Wiley-Liss, Inc.

Key words: *DLD*; pyruvate dehydrogenase; branched-chain ketoacid dehydrogenase; dihydrolipoamide dehydrogenase deficiency; E3 deficiency; lactic acidemia

## INTRODUCTION

The  $\alpha$ -ketoglutarate dehydrogenase complex (KGDC) catalyses the oxidative decarboxylation of  $\alpha$ -ketoglutarate ( $\alpha$ -KG) to succinyl-coenzyme A in the tricarboxylic acid cycle. This multienzyme complex is made up of three

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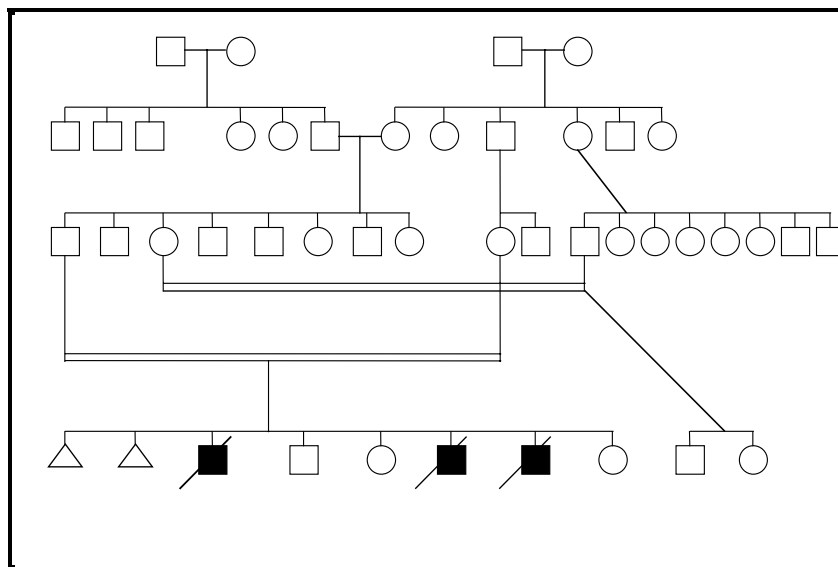
subunits: 2-oxoglutarate dehydrogenase (E1; HUGO symbol *PDHAI*; EC 1.2.4.2), dihydrolipoamide succinyltransferase (E2; HUGO symbol *DLST*; EC 2.3.1.61) and dihydrolipoamide: NAD<sup>+</sup> oxidoreductase (E3; HUGO symbol *DLSD*; EC 1.8.1.4) (Yeaman, 1989). The cDNAs specific to each subunit have been sequenced (Otulakowski and Robinson, 1987; Pons et al., 1988; Koike et al., 1992; Nakano et al., 1993) and the corresponding genes have been mapped (7p13-p14, 14q24.2-q24.3, and 7q31-q32, respectively) (Scherer et al., 1991; Nakano et al., 1993; Koike, 1995) and identified (Feigenbaum and Robinson, 1993; Nakano et al., 1994; Koike, 1995).

KGDC deficiency (MIM# 203740) is a rare autosomal recessive disorder, mostly characterized by neonatal onset, serious encephalopathy, and hyperlactatemia. KGDC deficiencies usually have a severe outcome leading to death in early infancy, and no efficient therapy is available so far. A few cases presenting either as hepatocellular injury without central nervous system involvement in adults (Barak et al., 1998) or as recurrent attacks of myoglobinuria (Elpeleg et al., 1997b) have also been reported. Accumulation of  $\alpha$ -KG and its metabolites in blood and urine is a key clue to the diagnosis. E3 deficiency (MIM# 246900), the most common among KGDC deficiencies, additionally promotes accumulation of pyruvate, and branched-chain aminoacids (BCAA) and their metabolites in blood and urine. KGDC E3 subunit is indeed common to two other enzymatic complexes, namely pyruvate dehydrogenase complex (PDC) and branched-chain ketoacid dehydrogenase complex (BCKDC). Diagnosis of KGDC deficiencies ultimately relies on enzymatic assay and when possible on the identification of the disease-causing gene mutation. Only few molecular studies have been hitherto reported with respect to E3 deficiency (Liu et al., 1993; Hong et al., 1996; Elpeleg et al., 1997a; Hong et al., 1997; Shaag et al., 1999; Shany et al., 1999; Cerna et al., 2001, Grafakou et al., 2003). We report here the enzymatic and molecular study of an atypical case of E3 deficiency in a sibship comprised of three affected individuals.

## PATIENTS AND METHODS

### Case report

Three affected male siblings whose clinical history has been detailed elsewhere (Bonnefont et al., 1992) were born to first-cousin Algerian parents.



**Figure 1.** Pedigree of the E3-deficient family.

Briefly, these children developed a truncal hypotonia with limb rigidity and choreoathetoid movements immediately after birth. Severity of the motor dysfunction contrasted with adequate emotional reactivity. Brain

scan showed mild cortical atrophy. In addition, patient 2 had a hypertrophic cardiomyopathy. Neurologic deterioration resulted in death at the age of 32, 30, and 13 months, respectively.

Laboratory tests were carried out in the three affected individuals, showing permanent hyperlactatemia (2.8-6.1 mM) with normal pyruvicemia (0.11-0.17 mM), increased lactate/pyruvate (L/P) molar ratio (23-46), and postabsorptive hyperketonemia (0.4-0.6 mM), with a normal 3 hydroxybutyrate/acetoacetate molar ratio. Increased level of blood glutamate with occasional excretion of  $\alpha$ -ketoglutaric acid in urine were suggestive of KGDC deficiency. Accordingly, the overall KGDC activity was found markedly deficient in cultured skin fibroblasts from the affected patients (Bonfont et al., 1992). E3 activity was suggested to be normal based on both the absence of accumulation of BCAA and their metabolites in plasma and urine, and a single E3 assay. However, the finding of a moderate decrease of PDC activity remained unclear at that time and prompted us to further investigate these patients.

### Enzyme assays

Fibroblasts were grown from a skin biopsy of the three patients.

Sub-confluent fibroblasts were harvested, washed twice with phosphate buffer saline and then centrifuged at 1500 g for 10 min. Cells were incubated 5 min on ice with Triton X-100 0.5 % and centrifuged at 15000 g for 5 min. Supernatant was preserved on ice for enzyme determination.

KGDC complex activity was spectrophotometrically measured as previously described (Robinson, 1987).

E1 activity was spectrophotometrically assayed as the reduction of FeCN and monitored at 420 nm for 15 min. The assay medium consisted of 40 mM  $\text{PO}_4$  (pH 6.5), 10 mM  $\text{MgCl}_2$  supplemented with 2 mM  $\alpha$ -KG, 0.8 mM TPP, 225  $\mu\text{M}$  FeCN.

E3 activity was monitored by the oxidation rate of NADH at 340 nm for 15 min in 1 ml of assay medium consisting in 40 mM  $\text{PO}_4$  (pH 7.5), 1.25 mM EDTA supplemented with 250  $\mu\text{M}$  NADH and 400  $\mu\text{M}$  LS. E3 measurement was made both in reverse ( $\text{NADH} + \text{H}^+ + \text{LS} \rightarrow \text{NAD}^+ + \text{LSH}_2$ ) and forward directions ( $\text{NAD}^+ + \text{LSH}_2 \rightarrow \text{NADH} + \text{H}^+ + \text{LS}$ ).

Assays of the enzyme activities were first carried out in control cell lines, with varying substrate concentrations (225-2250  $\mu\text{M}$  FeCN, 0.4-1.6 mM TPP for E1 assays ; 25-250  $\mu\text{M}$  NADH and 6.25-400  $\mu\text{M}$  LS for E3 assays) at different pH (6-7.5) and temperature conditions, in order to standardize the assay conditions. All spectrophotometric assays were performed at 37°C in 1 ml cuvettes.

Activities of BCKDC and PDC were assayed as described elsewhere (Wendel et al., 1975; Chretien et al., 1995).

Protein concentration was measured according to the method of Bradford.

### Nucleic acid analysis

gDNA from the patients, their parents, and 100 controls was extracted according to a standard method. RNAs were extracted from probands' fibroblasts according to Chomczynski's method (Chomczynski and Sacchi, 1987).

Microsatellites analysis : PCR primers were designed according to published data on E1 (Koike, 1995), E2 (Nakano et al., 1993), and E3 (Scherer et al., 1991) gene mapping, as following: D7S484 (7p15) and D7S506 (7p13-p14) for E1; D14S277, D14S577, and D14S574 (14q24) for E2; IVS6aGATT (Chehab et al., 1991), IVS8CA (Morrall et al., 1991), and IVS17BCA (Zielenski et al., 1991) (introns 6b, 8, and 17b of the CFTR gene, respectively, on 7q31) for E3. After DNA amplification (35 cycles; annealing temperature 55°C for D7S484, D7S506, D14S277, D14S577, D14S574 or 53°C for IVS8CA, IVS17BCA, and IVS6aGATT) in the three affected siblings and their parents. PCR products were migrated on a 6% polyacrylamide 7.5M urea gel, then transferred onto a Hybond N+ membrane (Amersham), hybridized with a (GT)<sub>10</sub> probe, and revealed by chemoluminescence (ECL kit, Amersham).

Southern Blot analysis : 5  $\mu\text{g}$  of gDNA from patient 1 were digested with either PstI, or EcoRI, or HindIII, electrophoresed on 1% agarose gel, transferred onto a Hybond N+ membrane (Amersham), and hybridized with the full-length E3 cDNA probe. The probe was generated from control RNAs by RT-PCR (Perkin Elmer kit) using

the “upstream fragment A-downstream fragment F” set of primers and was thereafter <sup>32</sup>P-labeled by random priming (Boehringer kit).

Northern Blot analysis: 5 µg of fibroblast total RNAs from patient 1 were electrophoresed on 1% agarose/formaldehyde/MOPS gel, transferred onto a Hybond N+ membrane (Amersham) that was sequentially hybridized with the full-length E3 cDNA and the actin cDNA as a reference probe.

Sequence analysis of the E3 cDNA (GenBank: NM\_000108.2): Total RNAs were reverse transcribed (Gene Amp RNA PCR kit, Perkin Elmer) using an oligodT primer. The full coding sequence of the E3 cDNA (1527 nucleotides) was subsequently amplified as six overlapping fragments. For each of the 6 fragments, first and second primers correspond to “sense” and “reverse” ones, respectively, as following: fragment A: TCCCAGCGGAGGTGAAAAGTAT (- 48) and CATCTTGCTCTAAATTCAGCGA (369); fragment B: GGCCCATGGAAAAGATTTTGC (300) and TTCATCTATCGTGATTCCAGG (591); fragment C: GCGGCACTCAGGTTATTGAT (505) and TGACTTCTTGGTAGCACCAGT (855); fragment D: CGCATCCTTCAAAAACAGGGG (787) and CAGCATTGGACCAGCAACTAC (1086); fragment E: CCAGATTTCAAATAAAATTCC (1022) and TTCACAGGATGCTCCATATTC (1434); fragment F: CAGGTGCTGGAGAAATGGTAA (1373) and TTCCAGGATGCTACAATAAGT (1775). All primers are given in the 5'→ 3' orientation. In parenthesis numbering denotes the position of the 5' end nucleotide of each primer. PCR reactions were carried out using the following conditions: 30 cycles; 1 µM primers, and 1.5 mM MgCl<sub>2</sub>; 94°C / 30 sec, 55°C / 30 sec, and 72°C / 1min. PCR products were sequenced according to the manufacturer's instruction, and sequencing products were analyzed on an automatic DNA sequencer (Applied Biosystems 373A).

**Restriction enzyme analysis:** gDNAs from the patients, their parents, and 100 controls were amplified (30 cycles; 1µM primers and 1.5 mM MgCl<sub>2</sub>; 94°C / 30 sec, 55°C / 30 sec, and 72°C / 1 min) using primers dedicated to fragment F amplification. PCR products were digested by *Bfal* or *Bpml* (New England Biolabs), according to the manufacturer's recommendations.

**Mutation nomenclature:** DNA numbering is based on cDNA sequence GenBank NM\_000108.2 as the reference, with nucleotide +1 corresponding to the A of the ATG translation initiation codon. Amino acid numbering is based both on the amino acid sequence of the native protein where amino acid 1 denotes the translation initiator Methionine of the unprocessed protein (reference nomenclature), and on the amino acid sequence of the processed gene product where amino acid 1 denotes the amino acid immediately following the leader peptide (first 35 amino acids of the native protein), as used for most E3 mutations hitherto reported.

## RESULTS

### Microsatellite analysis

Microsatellite analysis showed that patients 1 and 2 were haplodifferent at E1 and E2 loci as well (patient 3 not tested for these loci), while all three patients were homozygous and haploidentical at the E3 locus (7q31), consistent with a mutation of the E3 subunit gene (detailed data available on request).

### Enzymatic studies (Table 1)

When measured at saturating concentrations of each substrate in both forward (physiological) and reverse directions in the three patients, E1 activity was normal while E3 activity was about 20 % of control values when measured. Subsequent addition of control fibroblast homogenate resulted in the predictable control rate, ruling out any potential experimental artefact in our assay condition (not shown). The E3 Km for NADH and LS was identical in patients and controls (not shown). Residual activities of PDC and BCKDC were 63 % and 56 % of mean control values, respectively.

**Table 1. Enzymatic Activities of KGDC, KGDC E1 and E3 subunits, PDC and BCKDC in Fibroblasts from Affected Patients and Controls**

Activities	Patient 1	Patient 2	Controls (n)
KGDC*	< 0.01	< 0.01	11.2 ± 3.9 (7)
E1 subunit	3.3	4.1	3.8 ± 0.2 (3)
E3 subunit	5.6	7.4 and 7.2	24.8 and 35.7 (2)
PDC*	0.52	ND	0.82 ± 0.09 (6)
BCKDC	0.02	ND	0.036 ± 0.012 (14)

Enzymatic activities expressed as nmol/min/mg protein. Each assay was performed in duplicate. E3 activity was measured in reverse direction. (n): number of control cell lines tested. ND: not done; \*: values from Bonnefont et al., 1992.

### Mutation detection

Southern blot and Northern blot analyses, carried out in patient 1 only, failed to detect any large rearrangement of the E3 gene, or anomaly in size and/or steady-state amount of the E3 mRNA (not shown).

Full-length E3 cDNA from the three affected individuals was therefore sequenced. Compared to the control sequence, a homozygous A>G transition at nucleotide 1444 was detected in the three patients (reviewed but not shown). Since c.1444A>G abolished a BfaI restriction site and created a BpmI restriction site, the presence of this mutation was confirmed by PCR restriction at the genomic DNA level. It was found homozygous in the probands and heterozygous in their parents, while it was not detected in 100 individuals (reviewed but not shown). This nucleotide substitution, located within E3 exon 13 (Feigenbaum and Robinson, 1993), predicted a p.R482G substitution (or R447G in the processed gene product). Sequencing of all remaining coding regions of the E3 cDNA in the three patients revealed no other variation compared to the reference sequence (NM\_000108.2).

## DISCUSSION

We report the characterization of a KGDC deficiency previously identified in three siblings. According to the fact that dihydrolipoamide: NAD<sup>+</sup> oxidoreductase (E3) is the only enzymatic subunit common to KGDC, PDC and BCKDC, any combined deficiency of these enzymatic activities is suggestive of E3 deficiency. Preliminary enzymatic data (Bonnefont et al., 1992) showing a discrepancy between decreased activities of both KGDC and PDC, and the apparently normal E3 activity (compared to the value from a single control fibroblast line only), prompted us to carry out a haplotyping study with poly [AC] markers linked to KGDC E1, E2, and E3 loci. Linkage analysis strongly suggested that the disease was secondary to an E3 mutation. In agreement, E3 activity, measured with a more reliable test than the one devised in the initial clinical report (Bonnefont et al., 1992) was shown to be markedly deficient while E1 activity was normal. The c.1444A>G mutation, predicting a p.R482G substitution (or R447G in the processed gene product), is likely to be responsible for the disease. As expected from parental inbreeding, the three patients were indeed homozygous while their parents were heterozygous for this mutation, that was not found in one hundred controls tested. Furthermore, this mutation is predicted to substitute a small and neutral amino acid (Gly) for a large and basic amino acid (Arg) in a highly conserved domain of the protein (Table 2).

**Table 2. Comparison of Amino Acid Sequence of the E3 Protein Region Encompassing Amino Acid 447 Across Different Species**

Source	Amino acid sequence	Amino acids	Reference
<i>E.coli</i>	EMGCDAEDIALTIHAHPTLHES	430-451	Stephens, 1983
<i>P.sativum</i>	QYDASSEDIARVCHAHPTM	455-473	Bourguignon, 1992
<i>S.cerevisiae</i>	LALEYGASAEDVARVCHAHPTLSEA	439-463	Browning, 1988
<i>Murine</i>	EYGASCEDIARVCHAHPTL	437-455	Johnson, 1997
<i>Porcine</i>	LALEYGASCEDIARVCHAHPTLSEA	434-458	Otulakowski, 1987
<i>Human</i>	LALEYGASCEDIARVCHAHPTLSEA	434-458	Otulakowski, 1987; Pons,
<i>E3 patient</i>	LALEYGASCEDIAGVCHAHPTLSEA	434-458	1988 This study

Amino acid 1 denotes the amino acid immediately following the leader peptide (first 35 amino acids of the native protein).

In the literature, twenty-three patients with a KGDC deficiency, belonging to nineteen families, have been reported to date (see review by Robinson, 2001 and references below). These patients can be categorized into two groups, based on the involvement of either E2 or E3. Elevation of plasma pyruvate (L/P ratios normal or low) and accumulation of metabolites derived from BCAA, indicative of the simultaneous involvement of PDC (undetectable to 38 % of controls) and BCKDC overall activities (< 5 to 21% of controls), were found in all E3-deficient patients but one (Shany et al., 1999). Conversely, in our patients, absence of any biological clues to PDC and BCKDC dysfunction in vivo was in agreement with only “mild” PDC and BCKDC deficiencies, compared to control values (residual values of 63% and 56%, respectively). It can be hypothesized that, in our patients, residual activities of BCKDC and PDC are sufficient to prevent substrate (BCAA, pyruvate) accumulation at basal state, thus implying that the level of residual activity below which E3 becomes “rate-limiting” in the whole enzymatic process may vary among KGDC, BCKDC, and PDC. Another possibility (non-exclusive of the first one) would be that a given E3 mutation might differentially affect interactions between the different subunits of each  $\alpha$ -ketoacid dehydrogenase complexes. In agreement to this hypothesis, human p.R482 (R447 in the processed gene product) is likely to be located within the interface domain of E3, as suggested from cristallographic data from yeast E3 (Toyoda et al., 1998).

From a molecular point of view, eleven other E3 mutations have been reported to date (Table 3).

**Table 3. Mutations of the E3 Gene**

DNA level		Protein level		Protein domain**	Reference
Nucleotide	Exon/Intron	Unprocessed	Processed		
c.104_105insA	E 2	p.Y35X*	absence	Leader peptide	Hong, 1996; Elpeleg, 1997a; Shaag, 1999
c.214A>G	E 4	p.K72E	K37E	FAD-binding	Liu, 1993
c.405_407delAGG	E 6	p.G136del	G101del	FAD-binding	Hong, 1997
c.685G>T	E 9	p.G229C	G194C	NAD-binding	Shaag, 1999
IVS9+1G>A	I 9	absence	absence		Grafakou, 2003
c.1081A>G	E 11	p.M361V	M326V	Central	Cerna, 2001
c.1123G>A	E 11	p.E375K	E340K	Central	Hong, 1997; Cerna, 2001
c.1178T>C	E 11	p.I393T	I358T	Interface	Grafakou, 2003
c.1436A>T	E 13	p.D479V	D444V	Interface	Shany, 1999
c.1444A>G	E 13	p.R482G	R447G	Interface	This study
c.1463C>T	E 13	p.P488L	P453L	Interface	Liu, 1993
c.1483A>G	E 14	p.R495G	R460G	Interface	Hong, 1996

GenBank accession number: NM\_000108.2. The DNA mutation numbering is based on cDNA sequence with nucleotide +1 corresponding to the A of the ATG translation initiation codon. E: exon, I: intron. Amino acid 1 denotes i) the translation initiator Methionine of the unprocessed gene product, ii) the amino acid immediately following the leader peptide (first 35 amino acids of the native protein) of the processed gene product. \*\*FAD domain: amino acids 1-148, NADH-binding domain: amino acids 149-282, central domain: amino acids 283-351, interface domain: amino acids 352-474, by reference to data from yeast (Toyoda et al., 1998).

Including c.1444A>G (p.R482G; or R447G in the processed gene product), these twelve E3 mutations account for ten different genotypes in sixteen unrelated families (Table 4). Only three of these mutations [c.104\_105insA (p.Y35X, resulting in absence of the mature protein), c.685G>T (p.G229C; or G194C in the processed gene product), and c.1123G>A (p.E375K; or E340K in the processed gene product)] have been reported in more than one E3 mutant allele. c.104\_105insA was detected at heterozygous state in five unrelated patients affected with recurrent episodes of liver failure (Hong et al., 1996; Elpeleg et al., 1997a; Shaag et al., 1999). c.685G>T has been found in twelve of fourteen mutant E3 alleles from seven Ashkenazi Jewish families (Shaag et al., 1999), resulting in a late-onset disease with predominant hepatic failure and few neurological symptoms in homozygous individuals. c.1123G>A was detected at heterozygous state in two unrelated families (Hong et al., 1997; Cerna et al., 2001).

**Table 4. Genotypes of E3-Deficient Patients**

Protein level		Number of families	Reference
[p.K72E]	+ [p.P488L]	1	Liu, 1993
[p.Y35X]	+ [p.R495G]	1	Hong, 1996
[p.G136del]	+ [p.E375K]	1	Hong, 1997
[p.Y35X]	+ [?]	2	Elpeleg, 1997a
[p.Y35X]	+ [p.G229C]	2	Shaag, 1999
[p.G229C]	+ [p.G229C]	5	Shaag, 1999
[p.D479V]	+ [p.D479V]	1	Shany, 1999
[p.M361V]	+ [p.E345K]	1	Cerna, 2001
[p.I393T]	+ absence	1	Grafakou, 2003
[p.R482G]	+ [p.R482G]	1	This study

Amino acid 1 denotes the translation initiator Methionine of the unprocessed gene product. [NM\\_000108.2](#).

Few data are available with respect to the impact of E3 gene mutations on the E3 protein function. Site-directed mutagenesis and expression in *E.coli* (Kim and Patel, 1992) suggested that p.H487 (H452 in the processed gene product) and p.E492 (E457 in the processed gene product), two amino acids close to p.R482 (R447 in the processed gene product), are involved in the binding of dihydrolipoamide. Our finding that E3 Km for LS is not affected in p.R482G fibroblasts, suggest that p.R482 is not crucial for LS binding in human.

It has to be emphasized that the only other case of E3 deficiency without any branched chain derivatives in fluids (Shany et al., 1999) resulted from a mutation of an amino acid (p.D479V; or D444V in the processed gene product ) very close to the mutated p.R482 reported here, This suggests that interaction of this region of E3 with other subunits might differ among KGDC, PDC, and BCKDC. Strikingly, both clinical reports on p.D479V and p.R482G indicate that patients were affected with hypertrophic cardiomyopathy (Shany et al., 1999; Bonnefont et al., 1992), while such a cardiac involvement has not been reported in association with other E3 mutations.

In the future, delineation of the functional consequences of additional mutations should further highlight the relationships between structural domains and functions of the human E3 protein.

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#### REFERENCES

- Barak N, Huminer D, Segal T, Ben Ari Z, Halevy J, Kasper RT. 1998. Lipoamide dehydrogenase deficiency: a newly discovered cause of acute hepatitis in adults. *J Hepatol* 29: 482-484.
- Bonnefont JP, Chrétien D, Rustin P, Robinson B, Vassault A, Aupetit J, Charpentier C, Rabier D, Saudubray JM. 1992. Alpha-ketoglutarate dehydrogenase deficiency presenting as congenital lactic acidosis. *J Pediatr* 121: 255-258.
- Bourguignon J, Macherel D, Neuberger M, Douce R. 1992. Isolation, characterization, and sequence analysis of a cDNA clone encoding L-protein, the dihydrolipoamide dehydrogenase component of the glycine cleavage system from pea-leaf mitochondria. *Eur J Biochem* 204: 865-873.
- Browning KS, Uhlinger DJ, Reed LJ. 1988. Nucleotide sequence for yeast dihydrolipoamide dehydrogenase. *Proc Natl Acad Sci USA* 85: 1831-1834.
- Cerna L, Wenchich L, Hansikova H, Kmoch S, Peskova K, Chrastina P, Brynda J, Zeman J. 2001. Novel mutations in a boy with dihydrolipoamide dehydrogenase deficiency. *Med Sci Monit* 7: 1319-1325.
- Chehab FF, Johnson J, Louie E, Goossens M, Kawasaki E, Erlich H. 1991. A dimorphic 4-bp repeat in the cystic fibrosis gene is in absolute linkage disequilibrium with the  $\Delta F508$  mutation: implications for prenatal diagnosis and mutation origin. *Am J Hum Genet* 48: 223-226.
- Chomczynski P, Sacchi N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162: 156-159.
- Chretien D, Pourrier M, Bourgeron T, Séné M, Rötig A, Munnich A, Rustin P. 1995. An improved spectrophotometric assay of pyruvate dehydrogenase in lactate dehydrogenase-contaminated mitochondrial preparations from human skeletal muscle. *Clin Chim Acta* 240: 129-136.
- Elpeleg ON, Shaag A, Glustein JZ, Anikster Y, Joseph A, Saada A. 1997a. Lipoamide dehydrogenase deficiency in Ashkenazi Jews: an insertion mutation in the mitochondrial leader sequence. *Hum Mutat* 10: 256-257.
- Elpeleg ON, Saada A, Shaag A, Glustein JZ, Ruitenbeek W, Tein I, Halevy J. 1997b. Lipoamide dehydrogenase deficiency: a new cause for recurrent myoglobinuria. *Muscle Nerve* 20: 238-240.
- Feigenbaum AS, Robinson BH. 1993. The structure of the human dihydrolipoamide dehydrogenase gene (DLD) and its upstream elements. *Genomics* 17: 376-381.
- Grafakou O, Oexle K, van den Heuvel L, Smeets R, Trijbels F, Goebel HH, Bosshard N, Superti-Furga A, Steinmann B, Smeitink J. 2003. Leigh syndrome due to compound heterozygosity of dihydrolipoamide dehydrogenase gene mutations. Description of the first E3 splice site mutation. *Eur J Pediatr* 162: 714-718.

- Hong YS, Kerr DS, Craigen WJ, Tan J, Pan Y, Lusk M, Patel MS. 1996. Identification of two mutations in a compound heterozygous child with dihydrolipoamide dehydrogenase deficiency. *Hum Mol Genet* 5: 1925-1930.
- Hong YS, Kerr DS, Liu TC, Lusk M, Powell BR, Patel MS. 1997. Deficiency of dihydrolipoamide dehydrogenase due to two mutant alleles (E340K and G101del). Analysis of a family and prenatal testing. *Biochim Biophys Acta* 1362: 160-168.
- Johnson M, Yang HS, Johanning GL, Patel MS. 1997. Characterization of the mouse dihydrolipoamide dehydrogenase (Dld) gene: genomic structure, promoter sequence, and chromosomal localization. *Genomics* 41: 320-326.
- Kim H, Patel MS. 1992. Characterization of two site-specifically mutated human dihydrolipoamide dehydrogenases (His-452 --> Gln and Glu-457 --> Gln). *J Biol Chem* 267: 5128-5132.
- Koike K, Urata Y, Goto S. 1992. Cloning and nucleotide sequence of the cDNA encoding human 2-oxoglutarate dehydrogenase (lipoamide). *Proc Natl Acad Sci USA* 89: 1963-1967.
- Koike K. 1995. The gene encoding human 2-oxoglutarate dehydrogenase : structural organization and mapping to chromosome 7p13-p14. *Gene* 159: 261-266.
- Liu TC, Kim H, Arizmendi C, Kitano A, Patel MS. 1993. Identification of two missense mutations in a dihydrolipoamide dehydrogenase-deficient patient. *Proc Natl Acad Sci USA* 90: 5186-5190.
- Morral N, Nunes V, Casals T, Estivill X. 1991. CA/GT microsatellite alleles within the cystic fibrosis transmembrane conductance regulator (CFTR) gene are not generated by unequal crossingover. *Genomics* 10: 692-698.
- Nakano K, Matuda S, Sakamoto T, Takase C, Nakagawa S, Ohta S, Ariyama T, Inazawa J, Abe T, Miyata T. 1993. Human dihydrolipoamide succinyl transferase : cDNA cloning and localization on chromosome 14q24.2-q24.3. *Biochim Biophys Acta* 1216: 360-368.
- Nakano K, Takase C, Sakamoto T, Nakagawa S, Inazawa J, Ohta S, Matuda S. 1994. Isolation, characterization and structural organization of the gene and pseudogene for the dihydrolipoamide succinyltransferase component of the human 2-oxoglutarate dehydrogenase complex. *Eur J Biochem* 224: 179-189.
- Otulakowski G, Robinson BH. 1987. Isolation and sequence determination of cDNA clones for porcine and human lipoamide dehydrogenase. *J Biol Chem* 262: 17313-17318.
- Pons G, Raefsky-Estrin C, Carothers DJ, Pepin RA, Javed AA, Jesse BW, Ganapathi MK, Samols D, Patel MS. 1988. Cloning and cDNA sequence of the dihydrolipoamide dehydrogenase component of human  $\alpha$ -ketoacid dehydrogenase complexes. *Proc Natl Acad Sci USA* 85: 1422-1426.
- Robinson BH. 1987. An enzymatic approach to the study of the Krebs tricarboxylic acid cycle. In : Darley-USmar VM, Rickwood D, Wilson MT, editors. *Mitochondria : a practical approach*. Oxford: IRL Press. p 153-170.
- Robinson BH. 2001. Lactic acidemia: Disorders of pyruvate carboxylase and pyruvate dehydrogenase. In: Scriver CR, Beaudet AL, Sly WS, Valle D, editors. *The metabolic and molecular bases of inherited disease*. Mc Graw Hill, 8<sup>th</sup> edition. p 2275-2295.
- Scherer SW, Otulakowski G, Robinson BH, Tsui LC. 1991. Localization of the human dihydrolipoamide dehydrogenase gene (DLD) to 7q31-->32. *Cytogenet Cell Genet* 56: 176-177.
- Shaag A, Saada A, Berger I, Mandel H, Joseph A, Feigenbaum A, Elpeleg ON. 1999. Molecular basis of lipoamide dehydrogenase deficiency in Ashkenazi Jews. *Am J Med Genet* 82: 177-182.
- Shany E, Saada A, Landau D, Shaag A, Hershkovitz E, Elpeleg ON. 1999. Lipoamide dehydrogenase deficiency due to a novel mutation in the interface domain. *Biochem Biophys Res Commun* 262: 163-166.
- Stephens PE, Lewis HM, Darlison MG, Guest JR. 1983. Nucleotide sequence of the lipoamide dehydrogenase gene of *Escherichia coli* K12. *Eur J Biochem* 135: 519-527.
- Toyoda T, Suzuki K, Sekiguchi T, Reed LJ, Takenaka A. 1998. Crystal structure of eucaryotic E3, lipoamide dehydrogenase from yeast. *J Biochem* 123: 668-674.
- Wendel U, Wentrup A, Rudiger HW. 1975. Maple syrup urine disease: analysis of branched chain ketoacid decarboxylation in cultured fibroblasts. *Pediatr Res* 9: 709-717.

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Yeaman SJ. 1989. The 2-oxo acid dehydrogenase complexes : recent advances. *Biochem J* 257: 625-632.

Zielenski J, Markiewicz D, Rininsland F, Rommens J, Tsui LC. 1991. A cluster of highly polymorphic dinucleotide repeats in intron 17b of the cystic fibrosis transmembrane conductance regulator (CFTR) gene. *Am J Hum Genet* 49: 1256-1262.