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Deficiency of dihydrolipoamide dehydrogenase due to two mutant alleles (E340K and G101del) Analysis of a family and prenatal testing

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Abstract

A male child with metabolic acidosis was diagnosed as having dihydrolipoamide dehydrognase (E3) deficiency. E3 activity of the proband's cultured fibroblasts and blood lymphocytes was 3–9% of normal, while in the parent's lymphocytes it was about 60% of normal. The proband's pyruvate dehydrogenase complex (PDC) and the α-ketoglutarate dehydrogenase complex activities from cultured skin fibroblasts were 12% and 6% of normal, respectively. PDC activity in the parents cultured fibroblasts was 25–31% of normal. Western and Northern blot analyses showed similar quantities of E3 protein and mRNA in cultured fibroblasts from the proband and his parents. DNA sequencing of cloned full-length E3 cDNAs, from the proband and the parents, showed two mutations on different alleles of proband were inherited from the parents. One mutation is a three nucleotide (AGG) deletion, from the mother, resulting in deletion of Gly101 in the FAD binding domain. The other mutation is a nucleotide substitution (G to A), from the father, leading to substitution of Lys for Glu340 in the central domain. The same deletion mutation was found in E3 cDNA from a chorionic villus sample and cultured fibroblasts obtained from the mother's subsequent offspring. This finding illustrates the possiblilty of successful prenatal diagnosis of E3 deficiency utilizing mutations characterized prior to initiation of pregnancy. © 1997 Elsevier Science B.V.

Keywords: Dihydrolipoamide dehydrogenase deficiency; Prenatal diagnosis; Inborn error; Pyruvate dehydrogenase complex deficiency; Multiple complex deficiency

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1. Introduction

Mammalian dihydrolipoamide dehydrogenase (E3, dihydrolipoamide:NAD^+ oxidoreductase, EC 1.8.1.4) is the common flavoprotein component of the three α-keto acid dehydrogenase multienzyme complexes, pyruvate dehydrogenase complex (PDC), α-ketoglutarate dehydrogenase complex (KGDC), and branched-chain α-keto acid dehydrogenase complex (BCKDC) [1]. E3 is also a component (known as the L protein) of the glycine cleavage system [2]. The human E3 gene is located on chromosome 7, within bands q31–q32 [3]. This gene encodes a 509 amino acid protein, of which the N-terminal 35 amino acids form a leader sequence having an α-helical structure that is cleaved during import into mitochondria [4]. The active enzyme is a homodimer of 51 kDa subunits with 4 distinctive subdomain structures (FAD binding, NAD^+ binding, central, and interface domains) and has 2 identical active sites composed of specific amino acid residues from both subunits. During catalysis, electrons are sequentially transferred from a dihydrolipoyl moiety bound to the substrate protein to the redox disulfide in the E3 protein, the FAD cofactor, and finally to NAD^+ to generate NADH.

Because E3 is the common component in these complexes, lack of functional E3 causes reduction in the activities in PDC, KGDC, and BCKDC [1]. E3 deficiency leads to development of lactic acidosis and neurological degeneration as well as elevated levels of plasma branched-chain amino acids and urinary excretion of the respective α-keto and α-hydroxy acids [5]. E3 deficiency has been well documented in at least 15 patients [6–15], but specific mutations in the coding region have been reported in only three patients [16–18]. In this paper, we report 2 additional mutations, a deletion and a substitution, together causing E3 deficiency and separately causing partial deficiency in the heterozygous parents.

2. Clinical history

The proband was a male child who was the product of a 42 week pregnancy, with a birth weight of 3.6 kg. He appeared normal after birth until the 3rd day when he developed seizures, hypoventilation, and circulatory failure associated with hepatomegaly, hypoglycemia, metabolic acidosis (pH 7.05), and elevated lactate (12.8 mM, normal: 0.6–2.5). He improved with i.v. glucose, bicarbonate, and frequent feeding. He then remained stable (lactate 2–4 mM) until age 2 months when he was hospitalized again for poor feeding and somnolence; his liver was not enlarged at that time. He developed lactic acidosis (10–16 mM) in the hospital while fasting. Serum amino acid analysis showed increased alanine (1348 μM; normal: 132–455), α-aminobutyrate (106 μM; normal: 4–24), leucine (300 μM; normal: 61–183), isoleucine (120 μM; normal: 27–80), valine (345 μM; normal: 78–142), glycine (553 μM; normal 106–254), threonine (236 μM; normal: 65–147), methionine (151 μM; normal: 6–36), and mildly increased phenylalanine, cystathionine, cysteine, and histidine. Urine organic acid analysis showed variable increases of lactic, pyruvic, and α-keto- and/or α-hydroxy-isovaleric, isocaproic, butyric, and glutaric acids, as well as succinic, adipic, suberic, and sebacic acids. While on a low carbohydrate diet (17% of energy as CHO, 65% fat, 17% protein), blood lactate was somewhat lower (4–6 mM). MRI of the head (age 19 months) was normal. At age 15 months, his length (50th%tile) and weight (25th%tile) were normal but his head was small (7th%tile). His development was delayed ($DQ = 50–67$). He developed progressive optic atrophy. D,L-Lipoic acid (50 mg/kg/d) was administrated for 4 months without apparent clinical or biochemical benefit. He died at age of 5 1/2 years following an acute illness. A limited autopsy was performed 2 h after death to obtain tissue samples, which were frozen.

The mother became pregnant again following the diagnosis of E3 deficiency in her son. Chorionic villus sampling was performed at approximately 6 weeks of gestation; part of this sample was frozen and assayed directly and another portion was cultured for growth of fibroblasts. She gave birth to a healthy female infant at 39 week gestation (birth weight 3.9 kg). There was no evidence in this child of any of the clinical problems which had affected her elder brother. Blood was obtained at birth from the umbilical cord for isolation of lymphocytes, and again at age 2 months; a skin biopsy was performed for growth of fibroblasts. This child has remained in

good health and is functioning normally at age of 7 years. The parents also have remained in good health and are functioning at high intellectual levels.

3. Materials and methods

3.1. Cell and tissue samples and enzyme assays

All samples from human subjects were obtained according to protocols approved by the Institutional Review Board of University Hospitals of Cleveland, with the informed consent of the parents of these two children. Isolation of lymphocytes, culture of fibroblasts, and assays of the activities of PDC, KGDC, and E3 were performed as previously described [17,19,20]. Control sample data were collected over several years through the Center for Inherited Disorders of Energy Metabolism, Case Western Reserve University, utilizing identical methods of sample collection and assays to those used for the results shown here; concurrent control samples were run with each assay.

3.2. Immunoblotting

14 μg of mitochondrial protein obtained from cultured fibroblasts of a normal control, the proband and his two parents were run on 12% sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis. The proteins were transferred to a nitrocellulose membrane using an electroblotting apparatus at 100 V for 1 h, and immunoblotting was done as previously described [17]. The E3 immunospecific bands were detected by E3 specific antibody raised against porcine E3 and by chemiluminescence (Du Pont). As a standard, 25 ng of highly purified human E3 was included.

3.3. RNA blotting

Total RNA was extracted from cultured fibroblasts of the patient and a control by the guanidium thiocyanate method [21]. 25 μg of total RNA was separated by electrophoresis in 0.8% agarose gel containing 50% formaldehyde and subsequently transferred to a Genescreen membrane [22]. The blot was prehybridized with 20 mM piperazine-*N*,*N*′-bis[2-ethanesulfonic acid], pH 6.4, 2 mM EDTA, 0.8 M NaCl, 50% formamide, and 100 mg/ml salmon sperm DNA at 42°C overnight. The membrane was probed with a 1.1 kb 5′ fragment of E3 cDNA labeled with [^{32}P]-α-dCTP by the random primer reaction [23]. The blot was washed with 2 × SSC/1% SDS, 1 × SSC/0.1% SDS, 0.1 × SSC/0.1% SDS at 50°C, air dried, and autoradiographed.

3.4. Construction of E3 cDNAs and DNA sequencing

E3 cDNA was prepared by reverse transcription and the polymerase chain reaction (RT-PCR) as previously described [17]. Total RNA was isolated from cultured fibroblasts of a control, the proband, the proband's younger sister, and the proband's parents as well as a frozen chorionic villus sample (10 mg) obtained from the mother's subsequent pregnancy, using the ''Perfect RNA'' isolation kit (5 Prime to 3 Prime). Reverse transcription was carried out for 1 h at 43°C in a 20 ml reaction mixture which contained 1 μg of total RNA, 1 μg of oligo d(T)$_{16}$ primer, 0.5 nmoles of dNTP mixture, 20 mM dithiothreitol, and 1 unit of SuperScript II reverse transcriptase (Life Technologies), in the reaction buffer supplied by the manufacturer. Using the oligo dT primed first strand cDNAs as templates, E3 cDNAs for each specimen were specifically amplified by PCR using a pair of primers (sense, 5′-GCGCGCGGATCCG-GAGGTGAAAGTATTGGCGG-3′; antisense, 5′-GCGCGCGGATCCTCAAAAGTTGATTGATTT-GCC-3′) that generate *Bam*HI restriction sites at both ends of the full-length E3 cDNA [17]. For the proband's E3 cDNA analysis, the amplified 1.5 kb fragment was isolated from agarose gel, cut with *Bam*HI, and ligated with pBluescript SK$^+$ vector digested with the same enzyme. After confirmation, the recombinant pBluescript vector containing E3 cDNA was transformed into *E. coli* XL1-Blue competent cells. Plasmids containing the appropriate size DNA inserts were sequenced 4 times using Sequenase kit ver. 2.0 (US Biochemicals), using 4 pairs of overlapping primers and pBluescript primers for the entire E3 cDNA. To confirm the proband's mutations and the inheritance of these mutations from the par-

Table 1
E3, PDC and CS activities in cells from the family[a]

Subject	Lymphocytes			Fibroblasts		
	E3	PDC	CS	E3	PDC	CS
Controls:						
Mean	67	1.68	52	58	2.47	36.2
3–97%tile	43–109	0.9–3.0	26–99	19–95	1.2–4.7	21–57
n	316	476	154	224	286	192
Proband[b]	6	0.22	59	2	0.30	17.4
%	9%	13%	113%	3%	12%	48%
Mother[b]	41	2.14	91	22	0.76	15.9
%	61%	127%	175%	38%	31%	44%
Father[b]	38	2.18	66	13	0.61	17.0
%	57%	130%	127%	22%	25%	47%
Sister[b]	26	1.30	48	15	0.70	17.0
%	39%	77%	92%	26%	28%	47%

[a] Activity expressed as nmol/min/mg protein and % mean of controls. (CS = citrate synthase).
[b] Each activity value shown is the average of from 4–12 replicate assays.
n = number of tested samples.

ents, direct DNA sequencing was performed with genomic DNAs of the proband and the parents in the regions surrounding the mutations.

4. Results

4.1. Enzyme assays

Assays of E3 in fresh blood lymphocytes and cultured skin fibroblasts from the proband were 9% and 3% respectively of the mean activity of controls (Table 1). In lymphocytes from his mother and father, E3 activities were 61% and 57% of controls, respectively, and in skin fibroblasts, E3 activities were 38% and 22% (Table 1). PDC activity was 13% of controls in lymphocytes from the proband but was not reduced in lymphocytes from the parents (127–130%). On the other hand, PDC activity was 12% of controls in cultured skin fibroblasts from the proband and well below the range of controls in fibroblasts from the mother and father (31% and 25% respectively). Therefore, the effect of partial E3 deficiency (in the parents) on PDC activity was more severe in cultured fibroblasts than in fresh lymphocytes. The activity of citrate synthase, used as an internal mitochondrial control, was about half of that in controls in skin fibroblasts from the proband and both of his parents (Table 1).

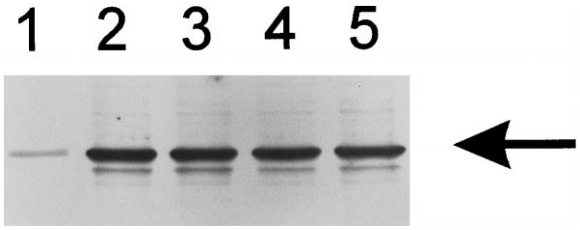

Fig. 1. Western blot analysis of E3 from cultured skin fibroblasts (14 μg of mitochondrial proteins) of control, the proband and his parents. Lane 1, purified human E3 (25 ng); lane 2, control; lane 3, proband; lane 4, father; lane 5, mother. The minor band, located under E3 band, appears to be E3 binding protein (protein X) [28].

KGDC activity was also greatly reduced in skin fibroblasts from the proband, corresponding to 6% of controls (Table 2). The relatively low activities of PDC and KGDC found in the proband's E3 deficient

Table 2
E3, PDC and KGDC activities in cultured fibroblasts and postmortem tissues from proband[a]

Subject	Fibroblasts			Heart			Skeletal Muscle			Liver		
	E3	PDC	KGDC	E3	PDC	KGDC	E3	PDC	KGDC	E3	PDC	KGDC
Controls:												
Mean	58	2.47	2.2	412	13.5	8.2	120	2.95	2.86	163	2.13	2.21
3–97%tile	19–95	1.2–4.7	0.5–5.2	179–598	7.6–24	4.8–11.8	63–204	1.1–7.3	1.5–4.8	92–281	1.1–4.2	0.5–3.5
n	224	286	23	25	25	6	161	173	9	49	51	5
Proband[b]	2	0.30	0.14	17.8	3	0.12	12.6	0.40	0.02	17	0.44	0.06
%	3%	12%	6%	4%	22%	1%	11%	14%	1%	10%	21%	3%

[a] Activity expressed as nmol/min/mg protein and % mean of controls.
[b] Each activity value shown is the average of from 2–8 replicates.
n = number of tested samples.

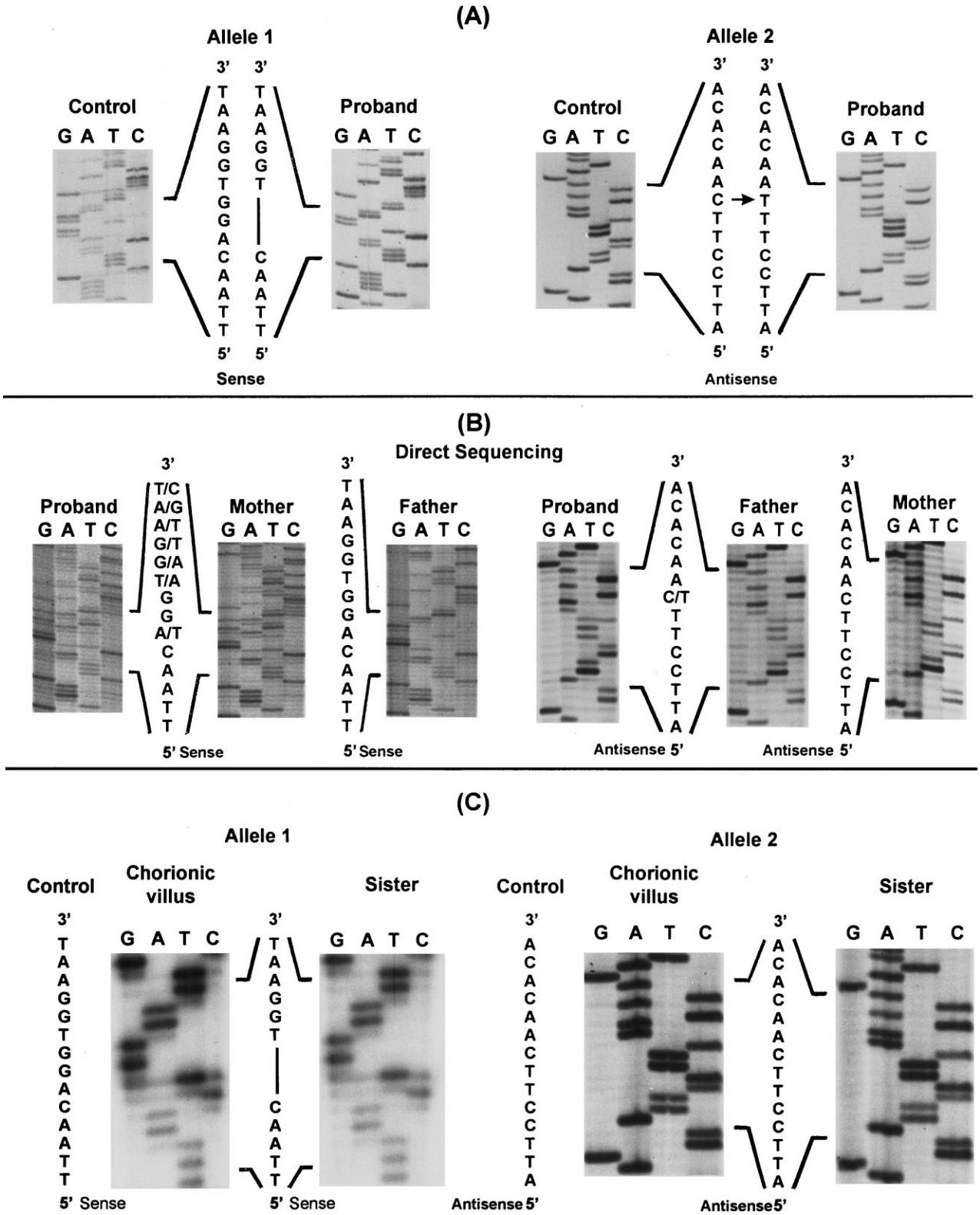
(A)
Allele 1
Control
G A T C
3' T A A G G T G G A C A A T T 5'
3' T A A G G T C A A T T 5'
Proband
G A T C
Sense
Allele 2
Control
G A T C
3' A C A C A A C T T C C T T A 5'
3' A C A C A A T T T C C T T A 5'
Proband
G A T C
Antisense
(B)
Direct Sequencing
Proband
G A T C
3' T/C A/G A/T G/T G/A T/A G G A/T C A A T T
Mother
G A T C
5' Sense
3' T A A G G T G G A C A A T T
Father
G A T C
5' Sense
Proband
G A T C
3' A C A C A A C/T T T C C T T A
Father
G A T C
Antisense 5'
3' A C A C A A C T T C C T T A
Mother
G A T C
Antisense 5'
(C)
Allele 1
Control
3' T A A G G T G G A C A A T T 5'
Chorionic villus
G A T C
Sister
3' T A A G G T C A A T T 5'
G A T C
5' Sense
5' Sense
Allele 2
Control
3' A C A C A A C T T C C T T A 5'
Antisense 5'
Chorionic villus
G A T C
Sister
3' A C A C A A C T T C C T T A 5'
G A T C
Antisense 5'

Table 3
E3 and PDC activities in chorionic villus samples[a]

Subject	Chorionic villus homogenate		Cultured chorionic villus fibroblasts	
	E3	PDC	E3	PDC
Controls:				
Mean	208	2.9	83	3.7
Range	154–302	1.2–6.3	67–98	2.2–5.9
n	5	14	5	12
Sister[b]	93	2.7	53	2.7
%	45%	93%	64%	73%

[a] Activity expressed as nmol/min/mg protein and % mean of controls.
[b] Each activity value shown is the average of from 2–8 replicate assays.
n = number of tested samples.

cultured skin fibroblasts are consistent with other tissues assayed. In heart, skeletal muscle, and liver samples obtained very soon after death, E3 activity was 4–11% of controls, PDC activity was 14–22%, and KGDC activity was only 1–3% (Table 2). In all cells and tissue samples assayed, the effect of E3 deficiency was greater on KGDC than on PDC.

4.2. Western and Northern blot analyses

The results of immunoblotting demonstrate that there was no difference in the content of E3 in cultured skin fibroblasts from the proband or his parents compared to that of a control subject (Fig. 1). Despite the loss of E3 activity, the level of expressed E3 protein seems not to be changed in the proband and his parents. Although immunoreactive E3 was present in cultured skin fibroblasts, this was not the case for postmortem liver, heart, or skeletal muscle samples, in which no E3 was detectable by immunoblotting (data not shown). The difference presumably reflects the variable stability of this mutant form of E3 in cultured cells vs. postmortem tissues, as ordinarily E3 is relatively stable postmortem.

RNA blot analysis showed double bands of E3 mRNAs which were 2.2 and 2.4 kb in size from both the proband's and control cells; there was no significant difference in the intensity of these bands between the subjects (results not shown). These findings indicate that E3 deficiency was not caused by a lack of E3 mRNA.

4.3. DNA sequencing of E3 cDNAs

Two mutations were found in separate clones of the proband's full-length E3 cDNA coding sequence (Fig. 2(A)). One allele of the patient's E3, found in 7 separate clones, showed a 3 nucleotide deletion (455th to 457th, AGG; third nucleotide of 100th codon for threonine and first two nucleotides of the 101st codon for glycine of the normal E3 coding sequence) resulting in the deletion of Gly101. The other allele of the proband's E3, found in 6 separate clones, had a substitution of A for G (T for C in the antisense sequence) at the 1173rd nucleotide, which results in substitution of the codon for Glu340 (GAA) with Lys340 (AAA). DNA sequencing of the proband's E3 cDNA was repeated 3 times for each RT-PCR generated product, and 3 independent RT-PCR reactions were performed to eliminate any possible Sequenase or polymerase error. In both the proband's

Fig. 2. DNA sequencing analysis of cloned and genomic E3 DNAs from control and 5 subjects. Only areas with mutations are shown. 'Allele 1' and 'Allele 2' are used to distinguish the two types of cloned E3 cDNAs, based on DNA sequencing. (A) Comparison of DNA sequences of cloned E3 cDNAs around the mutation between a control and the proband. Deleted nucleotides are designated with a solid bar. The substituted nucleotide in the proband is shown with an arrow. (B) Direct genomic DNA PCR sequencing of E3 gene from the proband and his parents. Since a three nucleotide deletion mutation caused a frame shift of DNA sequences from both the proband and his mother, overlapping sequences are shown in parts. For the substitution mutation found in both the proband and his father, only one overlapping sequence is identified. (C) DNA sequencing data of cloned E3 cDNAs from a chorionic villus sample and cultured skin fibroblasts from the proband's sister compared with a control.

and the mother's genomic DNAs, direct sequencing showed overlapping DNA sequences starting from the 455th nucleotide caused by deletion of 3 nucleotides in one of the two alleles, indicating that the deletion mutation was inherited from the mother (Fig. 2(B)). In direct sequencing of both the proband's and father's genomic DNAs, both G and A (C and T in the antisense sequence) bands were found at the 1173rd nucleotide, demonstrating that the substitution mutation was inherited from the father (Fig. 2(B)).

4.4. Analysis of prenatal samples

Direct assay of E3 in a homogenate of the frozen chorionic villus sample from the mother's subsequent pregnancy showed activity of 45% of controls, while PDC activity was 93% (Table 3). Later, fibroblasts cultured from this chorionic villus sample were assayed and showed E3 and PDC activities equivalent to 64% and 73% of controls, respectively (Table 3). In lymphocytes obtained from cord blood and at age 2 months, E3 was 39% of controls, whereas PDC activity was normal (77% of controls), while in cultured skin fibroblasts from this child, E3 activity was 26% of controls and PDC activity was 28% (Table 1). These assays of E3 activity in samples from the daughter are consistent with a heterozygous condition. The difference between the consequences of reduced E3 activity on PDC in lymphocytes and fibroblasts is similar to what was found in the parents. Citrate synthase activity was also lower in fibroblasts.

Mutational analysis of cloned E3 cDNA prepared from the frozen chorionic villus sample showed the same deletion mutation as found in the mother's E3 (missing 3 nucleotides AGG, from 455th to 457th, resulting in the deletion of Gly101) (Fig. 2(C)). This mutation was also confirmed in E3 cDNA from cultured skin fibroblasts from the sister (Fig. 2(C)).

5. Discussion

The severe clinical consequences in this child with a fatal form of E3 deficiency have been described in most of the other reported cases of this disorder [6–12,24,25]. While there is considerable clinical (and probably genetic) heterogeneity within this small population, the predominant phenotypic consequences are neurological, including impaired mental development, seizures, and degenerative deterioration [26]. The predictable metabolic manifestations of increased lactic, pyruvic, α-ketoglutaric, and branched-chain α-keto and α-hydroxy organic acids were all present, but the metabolic findings in this case were complex, which also has been true for other cases of E3 deficiency. In contrast to one previous case who appeared to benefit from administration of D,L-lipoic acid [9], there was no evidence for a beneficial effect of use of this agent in the present case.

The partial reduction of E3 activity in the heterozygous parents and sister has a less severe effect on PDC activity in blood lymphocytes or the chorionic villus sample than in cultured fibroblasts. Reduction of PDC activity has been observed previously in skin fibroblasts from E3 heterozygotes [17,27]. This difference may be due to the normally higher ratios of E3 to PDC activity in lymphocytes (40:1) and chorionic villus (72:1) than in fibroblasts (23:1). The lack of excess E3 in fibroblasts may be related to the observation that citrate synthase is also lower in these deficient and heterozygous cells, but the mechanism is not known.

In this paper, we have identified two new mutations, a 3 base pair deletion and a base substitution, in the E3-deficient male proband. These two mutations are located on different alleles. These mutations are significant because they affect the catalytic function of the E3 protein without affecting its content (Fig. 1) or the content of E3 mRNA in cultured skin fibroblasts. We found the mutations were inherited from heterozygous parents. Those findings depended on systematic characterization of full-length E3 cDNAs generated by RT-PCR from RNA isolated from cultured fibroblasts. DNA sequencing data and significantly reduced activities of E3, PDC and KGDC in the patient as well as a moderate reduction in the parent's cells confirm that these mutations were inherited from the parents in an autosomal recessive manner (Table 1).

The mutations found in this patient, which affect conserved amino acids, are located in the α-helixes of the FAD domain (Gly101del) and the central domain (Glu340Lys), based on amino acid homology

among E3s from various species (Fig. 3). The deletion mutation inherited from the mother (Gly101), located within the highly conserved amino acid residues of the 3rd α-helix of the FAD binding domain, probably causes a regional structural change which may affect the binding of FAD, resulting in the loss of E3 activity. Glycine, a small, non-polar residue, is not likely to be involved in inter-side chain relationships. The substitution mutation (Glu340Lys), inherited from the father, replaces a smaller, negatively charged side chain of an amino acid in a conserved α-helix with a bulky, positively charged side chain. Since Glu340 is located in the hydrophilic side of the α-helix (Fig. 3), this reversal in charge of the side chain would be expected to exert more effect on E3 activity than a local structural change due to the size difference between the two amino acids. Reduced E3 activity in fibroblasts from the father indicates that this substitution mutation causes a significant reduction (about 50%) of E3 activity. Based on the available X-ray crystal structure of E3 from three prokaryotic sources [29–31], it is not possible to discern the functions of these two amino acids in human E3 at the present time. The characterization of overexpressed recombinant mutant E3s would shed light on the possible roles of these two amino acids in human E3.

The heterozygous status of the proband's sister was detected in utero by assay of E3 activity in a chorionic villus sample and confirmed at birth in cord blood lymphocytes, as well as cultured fibroblasts from the chorionic villus and skin biopsies. To our knowledge, this is the first reported example of successful prenatal diagnostic testing for E3 deficiency.

(A)

Source	Amino acid sequence ↓	A.A. No.	Amino acid sequence ↓	A.A. No.
P. fluorescens	-KANIVKNLTGGIATLFKAN-	94-110	-SEEGVMVAERIAGHKAQM-	330-347
P. putida	-KDGIVDRLTTGVAALLKKH-	91-107	-MAQGEMVAEIIAGKARRF-	317-334
A. vinelandii	-KDQIVRNLTGGVASLIKAN-	94-110	-SEEGVVVAERIAGHKAQM-	330-347
E. coli	-KEKVINQLTGGLAGMAKGR-	89-105	-VHEGHVAAEVIAGKKHYF-	324-341
S. cerevisiae	-KDDAVKQLTGGIELLFKKN-	91-107	-EEEGIAAVEMLKTGHGHV-	337-354
Pig	-KSNAVKALTGGIAHLFKQN-	92-108	-EDEGIICVEGMAGGAVHI-	332-349
Dog	-KSTAVKALTGGIAHLFKQN-	92-108	-EDEGIICVEGMAGGAVPI-	332-349
Human	-KSTAVKALTGGIAHLFKQN-	92-108	-EDEGIICVEGMAGGAVHI-	332-349
E3-proband	-KSTAVKALT*GIAHLFKQN-	92-108	-EDEGIICVKGMAGGAVHI-	332-349

(B)

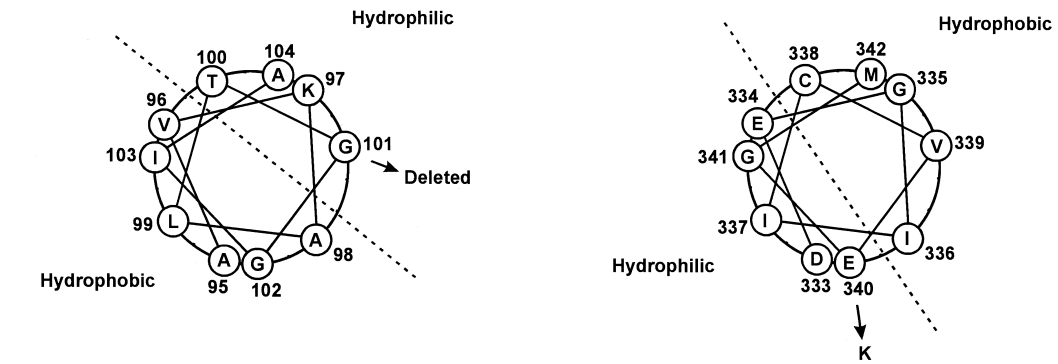

Fig. 3. Regional homology of E3 in the area of mutations and the pictorial representation of potential α-helical structures. (A) Amino acid sequence comparison of the area around two mutations in highly conserved regions, one (Gly101del) in the FAD binding domain and the other (Glu340Lys) in the central domain from various species. Arrows designate the site of mutations. An asterisk represents deletion of an amino acid (Gly101). The numbers on the right side of the amino acid sequences represent the locations of the first and last amino acid residues in the sequences. References for these sequences are: *P. fluorescens*, [29]; *P. putida*, [30]; *A. vinelandii*, [31]; *E. coli*, [32]; *S. cerevisiae*, [33]; pig, [34]; dog, [35]; human, [4]; E3-proband, (this paper). (B) Pictorial representation, in the Edmundson wheel projection, of the possible structures of two α-helices in which mutations were found. Only parts of the α-helices around the mutations are shown. Arrows indicate the sites of mutations.

After subsequently verifying the mutations present in the proband and his parents, DNA from the chorionic villus sample was analyzed and a single mutation was identified; this was confirmed in the daughter's cultured skin fibroblasts. Although in this case mutational analysis was done retrospectively, the same analysis could be done prospectively for other cases, if the two mutant alleles were characterized prior to conception, and would provide definitive corroboration of assay of E3 activity.

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