Maple Syrup Urine Disease

Complete Defect of the $E_1\beta$ Subunit of the Branched Chain α-Ketoacid Dehydrogenase Complex Due to a Deletion of an 11-bp Repeat Sequence which Encodes a Mitochondrial Targeting Leader Peptide in a Family with the Disease

Yoshitaka Nobukuni,* Hiroshi Mitsubuchi,* Izumi Akaboshi,* Yasuhiro Indo,* Fumio Endo,* Akira Yoshioka,‡ and Ichiro Matsuda*
**Department of Pediatrics, Kumamoto University Medical School, Kumamoto 860; and* ‡*Department of Pediatrics, Nara Medical University, Nara 634, Japan*

Abstract

Branched chain α-ketoacid dehydrogenase (BCKDH) deficiency results in maple syrup urine disease (MSUD). We examined the molecular basis of familial cases of MSUD by analyzing the activity, subunit structure, mRNA sequence, and genome structure of the affected enzyme. The BCKDH activity in the proband with MSUD was ~ 6% of the normal control level. Immunoblot analysis revealed that the $E_1\beta$ subunit of BCKDH was absent and that the $E_1\alpha$ subunit of BCKDH was markedly reduced. We amplified the cDNAs of the $E_1\alpha$ subunit and the $E_1\beta$ subunit of the BCKDH complex obtained from cells of the patient, using the polymerase chain reaction method, then sequenced the amplified cDNAs. The deduced amino acid sequence for the $E_1\alpha$ subunit of the patient's cell was normal. An 11-bp deletion was identified in the region that encoded the mitochondrial targeting leader peptide in the $E_1\beta$ cDNA. This 11-bp sequence is found in the first exon of the BCKDH-$E_1\beta$ gene, as a direct tandem repeat. Amplification of genomic DNA revealed that the consanguineous parents were heterozygous for this mutant allele, and sister and brother of the patient with the disease were homozygous for this mutant allele. This 11-bp deletion mutation caused a change in the reading frame and the mature $E_1\beta$ protein was defective. These observations show the biological importance of the $E_1\beta$ subunit of BCKDH to maintain normal function of the enzyme activity. The absence of the $E_1\beta$ subunit results in instability of the $E_1\alpha$ subunit. (*J. Clin. Invest.* 1991. 87:1862–1866.) Key words: gene mutation • complex enzyme • protein degradation • enzyme complex

Introduction

Branched chain α-ketoacid dehydrogenase (BCKDH)[1] (EC 1, 2, 4, 4) is a mitochondrial multienzyme complex catalyzing the oxidative decarboxylation of branched chain α-ketoacids derived from transamination of branched chain amino acids such as valine, leucine, and isoleucine (reaction 1).

$$\text{R-COCOOH} + \text{CoA-SH} + \text{NAD}^+ \xrightarrow{\text{TPP, } Mg^{2+}} \text{R-CO} \sim \text{S-CoA} + CO_2 + \text{NADH} + H^+. \quad (1)$$

The BCKDH complex consists of three catalytic components: branched chain α-ketoacid decarboxylase (E_1), dihydrolipoyl transacylase (E_2), and dihydrolipoamide dehydrogenase (E_3). E_1 is further composed of two subunits, $E_1\alpha$ and $E_1\beta$. E_1 and E_2 components are specific to BCKDH. The E_3 component is common among the three ketoacid dehydrogenase complexes, BCKDH, pyruvate dehydrogenase, and α-ketoglutarate dehydrogenase complexes. The BCKDH complex also contains two specific regulatory enzymes, a kinase and a phosphatase, which are responsible for regulating the catalytic activity through phosphorylation and dephosphorylation (1–3).

$E_1\alpha$ is the catalytic subunit phosphorylated at two serine residues and is responsible for regulating the catalytic activity, by covalent modification. E_2 catalyzes transfer of the acyl group from the lipoyl moiety to coenzyme A and forms the structural core of the enzyme complex and to this, E_1, E_3, kinase, and phosphatase are bound through noncovalent interactions. The function of $E_1\beta$ is unknown (1–3).

Impaired BCKDH activity leads to maple syrup urine disease (MSUD), an autosomal recessive inborn error of metabolism (1, 2). Etiology of MSUD is heterogeneous, as mutations in different regions of any of the BCKDH proteins could lead to the decreased functions of the entire complex. On the basis of clinical features several different phenotypes of MSUD have been elucidated, as follows, classical, intermediate, intermittent, and thiamine-responsive type (1, 2).

To elucidate the molecular mechanisms of MSUD, we and others have isolated and characterized cDNAs encoding human BCKDH-$E_1\alpha$ (4, 5), E_2 (6–9), and E_3 (10, 11). Recently, gene mutations of $E_1\alpha$ (12, 13) and E_2 (14) have been characterized clearly at the molecular level. To investigate the molecular mechanisms of the $E_1\beta$ deficiency, we isolated and characterized the cDNAs encoding the entire $E_1\beta$ subunit of the bovine (15) and of the human BCKDH complex (16) and we analyzed the genomic structure of the human BCKDH-$E_1\beta$ gene (Mitsubuchi, H., Y. Nobukuni, F. Endo, and I. Matsuda, manuscript in preparation).

Address reprint requests to Ichiro Matsuda, M.D., Ph.D., Dept. of Pediatrics, Kumamoto University Medical School, Honjo 1-1-1, Kumamoto 860, Japan.

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1. *Abbreviations used in this paper:* BCKDH, branched chain α-ketoacid dehydrogenase; MSUD, maple syrup urine disease.

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We discussed the possibility that $E_1\beta$ deficiency is one cause of the disease. In cases of $E_1\beta$ deficiency, immunologically cross-reactive material corresponding to BCKDH-$E_1\beta$ was not detectable (17, 18). We now report the molecular basis for familial cases of MSUD due to the $E_1\beta$ deficiency. Deletion of an 11-bp direct repeat sequence in exon 1 of the BCKDH-$E_1\beta$ gene became apparent and as a result of this mutation, mature $E_1\beta$ protein was entirely defective in the patients. The $E_1\beta$ subunit is required for stability of the $E_1\alpha$ subunit.

Methods

Cell lines and cell cultures. Lymphoblastoid cell lines were established by EBV-mediated transformation of peripheral blood B lymphocytes from the proband (19). Familial studies were done on peripheral leukocytes taken from 5–10 ml of (heparinized) blood samples from the family members.

Enzyme assay. Substrate-dependent kinetics of the BCKDH was determined for disrupted lymphoblastoid cells by quantifying the $^{14}CO_2$ released from α-keto [1-^{14}C] isovaleric acid, in the presence of co-factors, as described (17, 18).

Immunoblot analysis. Mitochondrial proteins were resolved by electrophoresis in a 10% polyacrylamide gel in the presence of SDS, essentially as described by Laemmli (20). The resolved proteins were electroblotted to nitrocellulose (21), and cross-reacting proteins were detected using affinity-purified rabbit anti-bovine BCKDH (E_1 + E_2) immunoglobulin, as described (17, 18).

Analysis of cDNA. Total RNA was isolated from cultured lymphoblastoid cells from the patient and from the peripheral lymphocytes of family members (22). First-strand cDNAs were generated from 30 μg of total RNA, using Moloney Murine Leukemia Virus reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD) in 50 mM Tris-HCl (pH 8.3), 40 mM KCl, 1 mM DTT, 6 mM $MgCl_2$, and 0.5 mM dNTPs (dATP, dCTP, dGTP, and dTTP) (12, 13, 23) with specific antisense oligonucleotides primers for $E_1\alpha$ or for $E_1\beta$. Two sets of sense/antisense oligonucleotides of each $E_1\alpha$ and $E_1\beta$ subunit were designed to cover the entire normal human cDNA sequence, as described (13). The cDNAs were then subjected to 30–40 cycles of enzymatic amplification (24, 25). The specific amplified cDNAs were subcloned into the multicloning site of a plasmid vector pUC18, and five independent clones of each amplified cDNA segment were sequenced (26, 27) using a Sequenase kit (United States Biochemical Corp., Cleveland, OH).

Analysis of genomic DNA. Genomic DNA was purified from cultured lymphoblastoid cells or peripheral leukocytes according to Kunkel et al. (28). Specific amplification of a genomic DNA which encompasses the region of the deletion detected in the mRNA of BCKDH-$E_1\beta$ subunit in the patient was attempted with oligonucleotide A (5′-CAGGGCGGCAGGGGCTGAGGGGCACT-3′) and B (5′-CCGGATCTGGCTGGAAAGTAAAATG-3′) (see Fig. 1 *a*). The amplified DNAs were subcloned into the multicloning site of pUC18, and five independent clones were sequenced, as described above.

Results

Clinical features of the MSUD patients. The proband E.K. (female) was the progeny of first cousin parents. Feeding difficulty began when she was 6 d old. Elevated leucine levels was detected at age 10 d by newborn screening for MSUD. Specific treatment was started immediately, however, opistotonus and hypertonia became evident at age 26 d. Then, peritoneal dialysis was initiated. Thereafter she was put on a protein and branched chain amino acids restricted diet. The phenotype was categorized as a classical type of MSUD. Her younger sister and brother were also diagnosed as cases of MSUD soon after birth and were also categorized as a classical type of the disease (see Fig. 1 *b*).

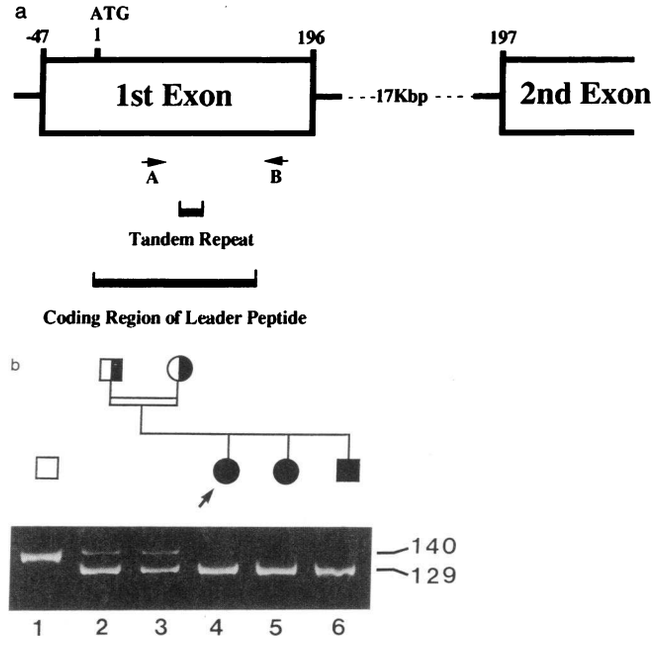

Figure 1. (*a*) Structure of part of the human BCKDH $E_1\beta$ gene and locations of the PCR primers. The open box depicts the first and second Exon of the BCKDH $E_1\beta$-gene. Solid arrows indicate the orientation and the region of primers used for PCR amplification. (*b*) Partial pedigree of the family and PCR analysis of the genomic DNA. All samples were amplified by PCR for the part of first Exon as described above. *1*, control; *2*, father; *3*, mother; *4*, proband; *5*, sister; *6*, brother.

Enzyme activity and immunoblot analysis. The rate of the overall reaction catalyzed by the BCKDH complex in the cultured lymphoblastoid cells from the patient (E.K.) was significantly reduced (~ 6% of the normal control). Immunoblot analysis of BCKDH revealed that three main immunogenic

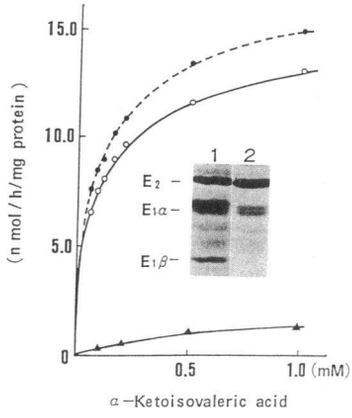

Figure 2. Activities of BCKDH and immunoblot analysis of BCKDH proteins in disrupted lymphoblastoid cells from disease-free and MSUD subjects. The rate of the overall reaction catalyzed by the multienzyme complex was measured as described in Methods. The cell lines from disease-free and MSUD subjects are control 1, control 2, the patients (E.K.). BCKDH immunoreactive proteins were detected by the immunoblot technique, using affinity purified antibody. The BCKDH (E_1 + E_2) peptides were, in decreasing size, E_2, dihydrolipoyl transacylase (M_r 52,000); $E_1\alpha$, the α-subunit of branched-chain α-keto acid decarboxylase (M_r 46,000); $E_1\beta$, the β-subunit of branched-chain α-keto acid decarboxylase (M_r 37,000). (Lane *1*) Disease-free cell line (control 1); (lane *2*) MSUD patient (E.K.).

peptides, the E_2 (M_r, 52,000), $E_1\alpha$ (M_r, 46,000) and $E_1\beta$ (M_r, 37,000) were present in the control cells (Fig. 2, lane *1*). In the patient (E.K.) cells, the $E_1\beta$ subunit was not detected and amounts of the immunological peptide of $E_1\alpha$ subunit were markedly reduced (Fig. 2, lane *2*).

Analysis of mRNA. To define the gene mutation in the patient (E.K.), cDNAs of the $E_1\alpha$ and $E_1\beta$ subunits of BCKDH were synthesized and amplified by reverse transcription of mRNA followed by PCR. Two sets of sense/antisense primers were designed for $E_1\alpha$ and $E_1\beta$ subunit, respectively, as described in Methods and based on the normal human BCKDH-$E_1\alpha$-cDNA and BCKDH-$E_1\beta$-cDNA. Thus, the cDNAs for the $E_1\alpha$ and $E_1\beta$ were amplified as two overlapping segments, respectively, and these were subcloned into pUC18 for DNA sequencing. The amplified segment corresponding to the 3′ region of $E_1\beta$-cDNA had a normal length and there was no evident change in the nucleotide sequence. Another amplified segment corresponding to the 5′ region of $E_1\beta$-cDNA was slightly shorter than the normal one. Nucleotide sequence analysis revealed an 11-bp deletion in the 5′ region, as shown in Fig. 3 *a*. This deletion removed nucleotides 80–90 (81–91) or 91–101 (92–102) of the cDNA (Fig. 3, *a* and *b*). This 11-bp sequence is found in the first exon of the normal gene of BCKDH-$E_1\beta$, as a direct tandem repeat. The amplified segment corresponding to the $E_1\alpha$-cDNA was of a normal length and there was no evident change in the nucleotide sequence.

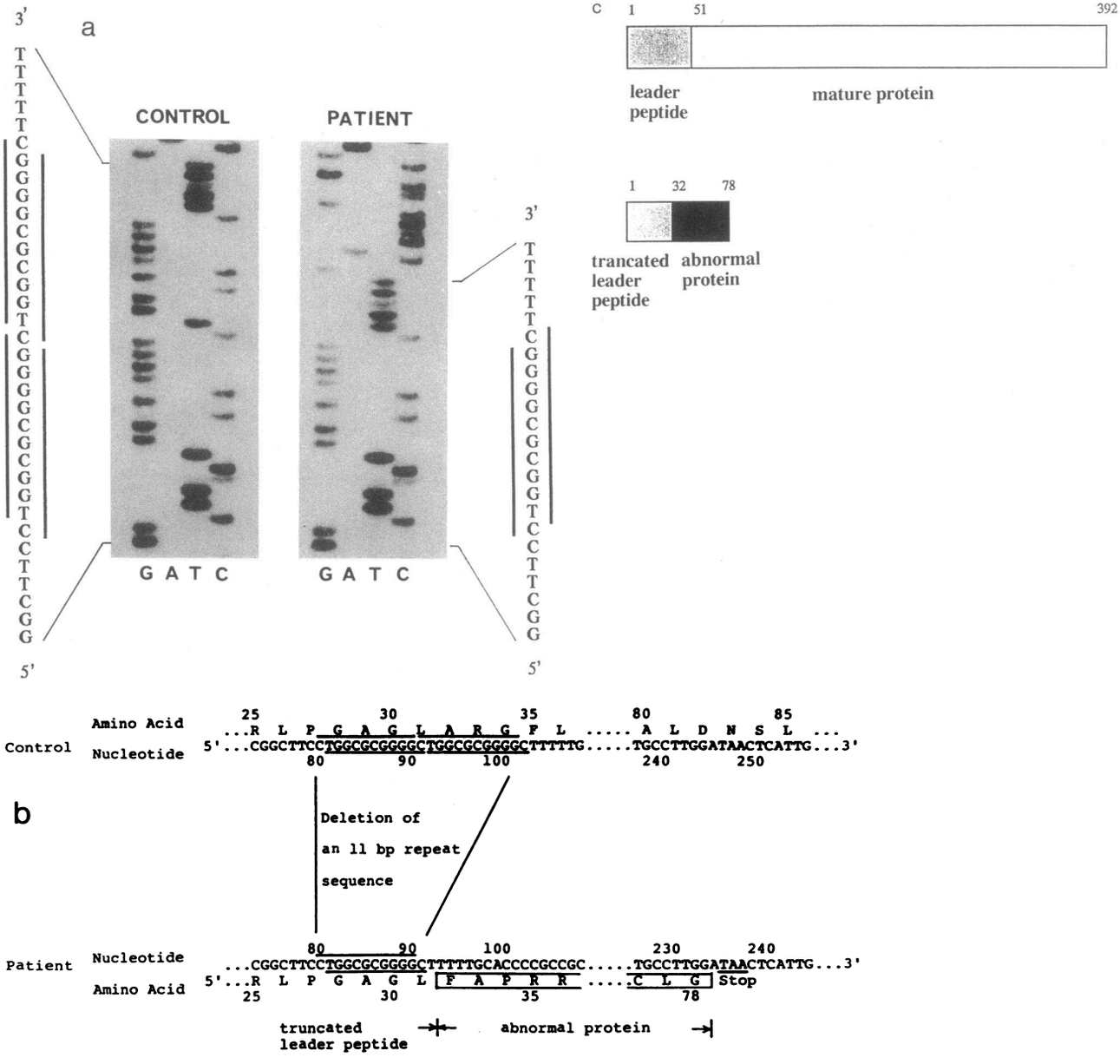

Figure 3. (*a*) Sequence analysis of cDNAs synthesized and amplified from mRNAs obtained from cultured cells from the patient and the control. Results of nucleotide sequencing of the normal cDNA and the mutant cDNA are shown. An 11-bp repeat sequence in the $E_1\beta$ gene was missing in this MSUD patient (E.K.). (*b*) Nucleotide and deduced amino acid sequence of the normal and the mutant $E_1\beta$ of BCKDH. (*c*) Schematic comparison of normal and the mutant $E_1\beta$ of BCKDH.

Gene analysis of the family. For further analysis, we amplified the genomic DNA from the patient and her family members. The parents had two species of PCR products, one with a normal size and one with a smaller size, a finding compatible with the abnormal DNA with a deletion of 11-bp nucleotides. The proband (E.K.), her sister, and her brother had only one product of a smaller size (Fig. 1 *b*). Analysis of the nucleotide sequence of the amplified DNA from the proband confirmed that the 11-bp sequence was deleted from the first exon of the BCKDH-$E_1\beta$ gene (data not shown).

Discussion

We have obtained what seems to be the first evidence for a molecular defect in the $E_1\beta$ subunit gene of BCKDH, as a cause of MSUD. An 11-bp deletion was identified in the region that encoded the mitochondrial targeting leader peptide of the $E_1\beta$ subunit. This deletion alters in the reading frame and the mature $E_1\beta$ protein seems to be entirely defective in this patient (Fig. 3, *b* and *c*). This deletion is part of a tandem 11-bp direct-repeat sequence. Repeat sequences are often involved in DNA rearrangements (29). Either "slipped mispairing" (30) or "unequal chromosome crossing-over" (31) was suggested to be the likely mechanism for generation of this mutation.

Despite the normal amino acid sequence for the $E_1\alpha$ subunit, the amount of the $E_1\alpha$ subunit, detected by immunoblot analysis, was reduced in the cells of this patient. It is most likely that the $E_1\alpha$ subunit is normally expressed but is rapidly degraded because of failure to assemble into a stable E_1 heterotetramer ($\alpha_2\beta_2$) accompanied with $E_1\beta$ subunit. Function of $E_1\beta$ subunit is not well understood (1–3), but it is apparently important for the stability of $E_1\alpha$ subunit.

We analyzed the molecular defect in cases of Mennonite MSUD (13) in which $E_1\beta$ was not detected and the amount of $E_1\alpha$ was reduced, as determined by immunoblot analysis (5, 17, 18). We noted a T-to-A missense mutation which changed tyrosine to asparagine at amino acid residue 394 of the $E_1\alpha$, in two different Mennonite MSUD patients (GM1655, GM1099), the amino acid sequence of the $E_1\beta$ subunit deduced from the cDNA from the patients was normal. It seems likely that the $E_1\beta$ subunit is normally expressed, but is rapidly degraded because of its failure to assemble in the stable E_1 ($\alpha_2\beta_2$) owing to mutation of $E_1\alpha$ in Mennonite MSUD.

All these studies clarified that gene defects in BCKDH-$E_1\alpha$ and BCKDH-$E_1\beta$ result in a similar biochemical phenotype, at the protein level. Both mutations, one with amino acid substitution on $E_1\alpha$ subunit and the other with total disappearance of mature $E_1\beta$, resulted in the defective activity of E_1 component and a closed phenotype of MSUD.

MSUD is a heterogeneous disorder affecting the function of BCKDH. As BCKDH is a multienzyme complex, mutations affecting different regions of any of the BCKDH subunits could lead to a decrease in the function of the enzyme complex. To clarify mechanisms involved in MSUD, measurements of the enzyme activity in cultured cells (32, 33), measurements of the generation time in cultured cells (33), complementation analysis (34, 35) and immunoblot analysis have been done (5, 17, 18, 36). However, in some cases it was difficult to identify which component of the BCKDH complex was primarily affected (5, 17, 18).

We analyzed findings in three patients with the classical type of MSUD, at the gene level and different mutations of $E_1\alpha$ (13), E_2 (14), and $E_1\beta$ (this study) were identified, in each case. The severity of the clinical symptoms seemed to be related to the magnitude of the subunit defect. Further gene analysis will be necessary to elucidate the molecular basis of other types of MSUD. These studies shed light on structural and functional relationships of components of the BCKDH complex.

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