Am. J. Hum. Genet. 68:232–237, 2001

Report

Gene Preference in Maple Syrup Urine Disease

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Untreated maple syrup urine disease (MSUD) results in mental and physical disabilities and often leads to neonatal death. Newborn-screening programs, coupled with the use of protein-modified diets, have minimized the severity of this phenotype and allowed affected individuals to develop into productive adults. Although inheritance of MSUD adheres to rules for single-gene traits, mutations in the genes for E1α, E1β, or E2 of the mitochondrial branched-chain α-ketoacid dehydrogenase complex can cause the disease. Randomly selected cell lines from 63 individuals with clinically diagnosed MSUD were tested by retroviral complementation of branched-chain α-ketoacid dehydrogenase activity to identify the gene locus for mutant alleles. The frequencies of the mutations were 33% for the E1α gene, 38% for the E1β gene, and 19% for the E2 gene. Ten percent of the tested cell lines gave ambiguous results by showing no complementation or restoration of activity with two gene products. These results provide a means to establish a genotype/phenotype relationship in MSUD, with the ultimate goal of unraveling the complexity of this single-gene trait. This represents the largest study to date providing information on the genotype for MSUD.

Maple syrup urine disease (MSUD; MSUD type Ia [MIM 248600], MSUD type II [MIM 248610], MSUD type Ib [MIM 248611]) is a rare inborn error of metabolism affecting <1 in 180,000 newborn infants in the general population (Peinemann and Danner 1994; Chuang and Shih 1995; Danner and Doering 1998). Despite its rare occurrence, MSUD is found in all racial and ethnic groups throughout the world (Danner and Elsas 1989). Neurological complications and death in the neonatal period can result from this inability to catabolize the branched-chain amino acids (BCAA) leucine, isoleucine, and valine. The pathophysiology of untreated MSUD is not well understood. Newborn-screening programs in most states and in several foreign countries detect those individuals at risk for expressing the disease. Affected individuals are protein intolerant and must be nurtured with synthetic diets that limit the intake of these essential BCAA (Elsas and Acosta 1988). These protein-modified diets enable near-normal growth and development for affected individuals but must be tailored to the individual, demonstrating one aspect of the complexity of this single-gene trait (Scriver and Waters 1999; Dipple and McCabe 2000).

The inability to catabolize the BCAA results from defective function of the branched chain α-ketoacid dehydrogenase (BCKD) complex. BCKD oxidatively decarboxylates the branched-chain α-ketoacids (BCKA) formed by the transamination of the BCAA. This mitochondrial multienzyme complex is encoded entirely by nuclear genes. Four protein products impart catalytic function, and two other protein products are used to regulate BCKD activity-state in the different tissues (Gillim et al. 1983; Wagenmakers et al. 1984). This regulation is necessary to prevent the depletion of BCAA, since BCKD is present in all cells. The need for this wide tissue distribution of BCKD is not understood.

Of the four genes that impart catalytic function, three encode proteins with specificity for the BCKA substrates. The fourth catalytic gene product also functions with other mitochondrial complexes (Yeaman 1989). Although inheritance of MSUD adheres to a simple autosomal recessive pattern, mutations in each of these three BCKD-specific genes have been shown to cause MSUD. Two of these genes encode subunits (E1α [Z14093] and E1β

Received October 9, 2000; accepted for publication November 6, 2000; electronically published December 7, 2000.

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0002-9297/2001/6801-0024$02.00

Table 1

BCKD Activity in Lymphoblast Lines with Defined Genotypes, from Patients with MSUD

Cell Line	Mutant Gene	Mutation	Mean ± SD Total BCKD Activity[a] (pmol $^{14}CO_2$/mg protein/3 h)			
			Not Transduced	E1α	E1β	E2
EM408	E1α	Y438N[b]	0 ± 0	166 ± 51	0 ± 0	0 ± 0
EM1952[c]	E1β	N176Y/R324X	24 ± 2	10 ± 1	364 ± 89	19 ± 6
EM3069[d]	E2	1031G→A/ (20-kb del)	71 ± 3	71 ± 14	82 ± 21	149 ± 43
DG75		None	6,158 ± 626	ND	ND	ND

[a] All cells were treated with 1mM α-chloroisocaproate for 10 min prior to addition of substrate to induce ability to measure full activity of the BCKD complex. ND = not done.
[b] Known Mennonite cell line.
[c] From McConnell et al. (1997).
[d] From Herring et al. (1992).

[M55575]) that form a heterotetramer that uses thiamin pyrophosphate as a cofactor to decarboxylate the BCKA (Ævarsson et al. 2000). The third gene product, the acyltransferase (E2 [X66785]) core protein, transfers the branched-chain acyl moiety to coenzyme A (Yeaman 1989; Reed and Hackert 1990).

The majority of mutations described to date have been in the gene that encodes the E1α subunit (Danner and Doering 1998). It remains to be shown whether any one of these three genes has an increased tendency toward mutation. Although all the genes have been characterized and their chromosomal location identified, no easy method is available to determine whether the E1α, E1β, or E2 gene harbors the mutant alleles. The only mutant allele found with any frequency exists in the Mennonite community and is associated with a founder effect (Danner and Doering 1998).

Finding a simple, reliable method to identify the specific mutant gene that causes MSUD from the three possible choices has presented a challenge. Western blots can be used to define individuals with MSUD who lack the E2 protein (Danner et al. 1985). However, reduction in the antigenic presence of E1α, E1β, or both can result from mutations in either gene, because the ability to form the $\alpha_2\beta_2$ tetramer is affected. In some MSUD cell lines, the proteins of the complex are antigenically indistinguishable from those seen in cells with normal activity. Thus, immunoblots have limited value in helping to identify the gene harboring the mutant alleles.

Enzyme-activity measurements in cultured cells derived from patients are used to confirm the clinical diagnosis of MSUD made by quantification of plasma BCAA concentration. We and others have shown that impaired BCKD activity is functionally restored to cultured cells by episomal addition of a wild-type cDNA for the defective gene (Litwer et al. 1989; Koyata et al. 1993; Mueller et al. 1995). Here we use this BCKD-activity-complementation approach to identify the gene harboring the mutant alleles. Retroviral plasmids holding the wild-type cDNA sequence were constructed for each of the three MSUD-specific genes in order to effectively and efficiently transduce both fibroblasts and lymphoblasts. Cell lines with defined mutations from individuals with MSUD were used to establish conditions for BCKD assay after transduction. As shown in table 1, the return of function to BCKD is clearly evident by 72 h after transduction of lymphoblasts and can specifically distinguish the defective gene despite an estimated transduction efficiency of only 30%. Production of the subunit encoded by the viral vector was demonstrated by western blot of mitochondrial protein from an E2-negative cell line transduced with the E2 and E1β vectors (fig. 1). Efficiency of transduction in fibroblast cultures is >70% by 48 h; thus, assays of BCKD activity can be performed at this time (table 2).

This enzyme-complementation analysis was applied to 63 cell lines from individuals with MSUD and without a known Mennonite ancestor. This represents the largest single study sample used for analysis of the MSUD genotype. No other qualification was placed on sample selection. The cell lines were obtained from physicians

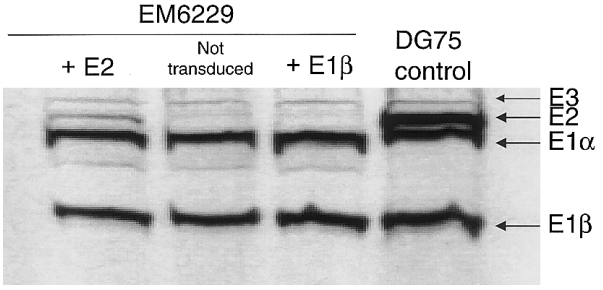

Figure 1 Western blot of BCKD proteins in mitochondria. Mitochondria were prepared from EM6229 cells without viral transduction or after transduction with either the E2 or E1β vector, and 20 μg were resolved for immunoblots. Mitochondria from DG75 cells serve as a wild-type control line.

Table 2

BCKD Activity in Fibroblast Lines with Unknown Genetic Mutations, from Patients with MSUD

Cell Line	Mean ± SD Total BCKD Activity[a] (pmol $^{14}CO_2$/mg protein/3 h)			
	Not Transduced	E1α	E1β	E2
EM15496	11 ± 4	2,437 ± 13	8 ± 0	10 ± 2
EM5621	1 ± 9	5 ± 0	2,128 ± 58	17 ± 1
EM4566	22 ± 3	29 ± 12	26 ± 4	2,112 ± 453
EM1118[b]	3,281 ± 113	ND	ND	ND

[a] Conditions for the assay are described in the text and in table 2. ND = not done.
[b] Control cell line.

throughout the world and included individuals from North, Central, and South America; Europe; and Asia. This sample includes 34 males and 29 females, in accordance with the autosomal recessive nature of MSUD. As depicted in figure 2*A*, mutations in the gene for E1β on chromosome 6 are approximately equal to the frequency of E1α mutations, whereas those of E2 are somewhat less common. In contrast, 52% of the previously characterized mutant alleles were the result of defects in the gene for the E1α subunit on chromosome 19, and 36% were defects in E2, as summarized in figure 2*B* (Chuang and Shih 1995; Danner and Doering 1998).

Results from five lymphoblastoid lines and one fibroblast line were ambiguous when this BCKD complementation analysis was used. For four cell lines, no stimulation of residual activity was observed with any subunit in three separate transductions for each cell line. The other two cell lines exhibited <10% increases in residual activity with two different subunits. Five of the six cell lines have residual activity >2% of wild-type BCKD activity, a condition that has been used to define an "intermittent" or "intermediate" form of MSUD (Peinemann and Danner 1994). In contrast, the majority of the other 57 cell lines have residual activity that is <2% of wild-type BCKD activity (table 3). Western blots of mitochondrial proteins from five of the six cell lines that gave ambiguous results show the antigenic presence in all subunits. One lacked the antigenic presence of both E1α and E1β. Another cell line that was found to be ambiguous in the lymphoblast assay was resolved when fibroblasts from the patient were analyzed; this was likely a result of the higher transduction efficiency for fibroblasts. Unfortunately, fibroblasts are not available for all patients studied. Definition of the specific mutations in the cells with ambiguous complementation data will resolve these questions and offer a clearer understanding of MSUD variability, especially regarding the "intermediate" phenotype (Peinemann and Danner 1994).

The somewhat lower frequency of mutations associated with the E2 gene on chromosome 1 may be real, but other explanations are possible. First, if the cell lines that give ambiguous results have mutations in the E2 gene, then an equal distribution of mutations among the three genes would result. A second explanation may be that the E2 mutations are being missed at the clinical level. As seen in table 3, individuals with E2 mutations can have higher residual activity than that found in cell lines with mutations in the other two genes. In some individuals there is no antigenic protein being produced (fig. 1) and yet some decarboxylation of the ketoacid substrate occurs (Danner et al. 1985; Ellerine et al. 1993). Mutations in E2 have also been associated with the "thiamin-responsive" form of MSUD (Danner et al. 1975; Fernhoff et al. 1985; Ellerine et al. 1993). One possible explanation for the thiamin response is that the E1 decarboxylase, which uses thiamin pyrophosphate as a cofactor in the reaction mechanism, can function independently of the E2 protein. The branched-chain acylthiamin pyrophosphate intermediate formed in the decarboxylation reaction is displaced by the excess thiamin pyrophosphate in the cell, thus allowing the enzyme to catalyze another round of decarboxylation (Herring et al. 1992; Danner and Doering 1998). The independent function of E1 could explain the higher residual activity observed for the E2 mutant cell lines. In some cases this activity could be high enough to prevent the full clinical phenotype, and therefore these individuals are never classified as having MSUD.

The ability of the enzyme complementation analysis to identify the mutant genes was confirmed by nucleotide sequence analysis of the indicated mutant gene with the primers listed in table 4. Results for one patient from each complementing group are reported in table 5. Parental cell lines for each proband were used to confirm the transmission of the mutant allele. The three examples all happened to be homozygous for the mutant allele, which is unusual for MSUD, since most probands thus far characterized are compound heterozygotes. Another

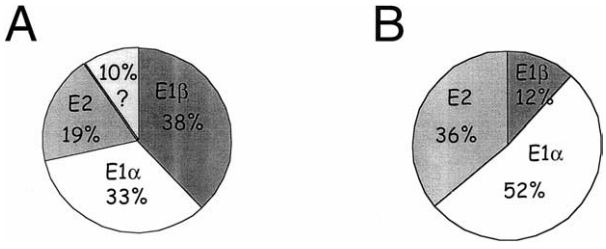

Figure 2 Percentage of cell lines with mutations in each of three genes that cause MSUD. *A*, Results of 63 cell lines using restoration of BCKD activity assay. Twenty-nine females and 34 males, excluding those with known Mennonite heritage, were tested. *B*, Summary of percentages based on mutations described elsewhere.

Table 3

Distribution of MSUD Cell Lines, Based on Mutant Gene and Percent Residual Activity of BCKD Above That in the Control Lines

	No. of MSUD Cell Lines in Which Residual Activity Compared with That in Wild-Type BCKD Is		
Mutant Gene	<2%	2%–10%	>10%
E1α	19	1	1
E1β	23	0	1
E2	9	3	0
Ambiguous[a]	1	3	2

[a] BCKD activity was complemented with more than one subunit, or there was no complementation.

unexpected result was that the mutant allele defined for the E1α gene in proband EM5579 was the allele that accounts for >99% of MSUD in the Mennonite population. It was previously thought that this allele was not found with greater frequency than other mutations in this gene in the general population. This proband's family has no known Mennonite heritage and the mating is not consanguineous. As more of the cell lines with mutations in this gene are defined, it will become possible to ascertain the true frequency of this mutant allele in the general population.

Mutations described in the E1β gene are for a proband who is the product of a consanguineous mating, of Turkish origin. The mutant allele results in a V251A substitution in the protein. According to the crystal structure coordinates (Ævarsson et al. 2000), the valine residues are on the surface of the $\alpha_2\beta_2$ tetramer, postulated to be in a region of interaction with the E2 core protein. It is not yet known how this alanine substitution in this position disrupts the function of BCKD (Ævarsson et al. 2000). A second Turkish proband (also the product of a consanguineous mating) who complemented with the E1β subunit was homozygous for the nucleotide change 1149T→A, introducing a stop codon at Y383. These results argue against the presence of a common allele within families of Turkish decent.

The homozygous mutation in the E2 gene in proband EM5591 produces an R462P amino acid substitution. At present it is not known whether this is a consanguineous mating. The R462 residue is just C-terminal to the putative coenzyme A binding site of the protein (Danner et al. 1989). The crystal structure for the E2 protein has not been solved, so it would be highly speculative to predict the way in which this amino acid substitution might change the ability to form the CoA ester.

The complex nature of single-gene traits has been attributed to variation in specific mutation sites, amino acid substitutions in the protein product, and the genomic background of affected individuals (Scriver and Waters 1999; Dipple and McCabe 2000). Variation in MSUD can also result from the fact that mutations in three different genes can produce a similar phenotype. The products of these genes must interact in a multienzyme complex to achieve their cellular function. To begin to unravel the complexity of MSUD we must first ask whether there is a variation in the occurrence of mutations in the three genes. Here we used this rescue technique to identify the defective subunit, and therefore the gene, with the mutant alleles. The method was effective in ⩾90% of cell lines tested. Previously, it was necessary to sequence all three genes and to exclude polymorphic changes. Now only one gene needs to be

Table 4

Primers Used to Prepare DNA for Nucleic Acid Sequence Analysis

Gene and Primer	Sequence (5′→3′)	Position in Coding Sequence	Annealing Temperature (°C)
E1α:			
Forward	CTAAACCGTGGTTTGAGCCA	32–52	54
Reverse	GCGTACGCCGCCCCCACCGCC	679–658	
Forward	GGCGGTGGGGGCGGCGTACGC	685–679	54
Reverse	GGTGGGGGTGGGCTGAGCAGG	1374–1353	
E1β:			
Forward	CGGGACTACGGGCAAACTCA	191–210	58
Reverse	AGAGCCCCATGACCAACACAGCCCC	628–604	
Forward	TGGCTACTCAGGCTCAGG	75–92	50
Reverse	TCCCCAGAGCGATAGCGATACTTG	565–542	
Forward	GGAATCGGAATTGCGGTCACTGGAG	444–468	58
Reverse	tcgcgaattcCAGTTACGTTAATGTCAGGGG	1286–1267	
E2:			
Forward	GCTGCAGTCCGTATGCTGA	18–36	54
Reverse	CTAGTAGCATAAAAGCTGGG	1456–1434	

Table 5

Nucleic Acid Changes in Mutant Genes Identified by BCKD Complementation Analysis

Cell Line	Mutant Gene	Mean ± SD Total BCKD Activity[a] (pmol $^{14}CO_2$/mg protein/3 h)		Nucleotide Change[b]	Amino Acid Change
		Baseline	Rescued		
EM5579	E1α	0 ± 1	136 ± 6	1312T→A	Y438N
EM608	E1β	37 ± 11	123 ± 18	752T→C	V251A
EM5591	E2	318 ± 54	569 ± 29	1385G→C	R462P

[a] BCKD activity is defined in table 2.

[b] All three probands were homozygous for the indicated change, and both parental lines in each family were heterozygous for the mutant allele.

scrutinized for nucleic acid changes. It appears that mutations occur in each of the three genes, and analysis of additional cell lines will allow a better estimation of the exact frequency. When amino acid substitutions result from these nucleic acid changes, it may be possible, in the future, to design specific reagents that will interact with the mutant protein in a way that alters the structure into a functional form. These reagents would offer a new and improved therapy for individuals with MSUD.

Acknowledgments

We thank all the physicians who have sent patient material to us over the past 20+ years. Without their help this study would not have been possible. Special thanks are given to Dr. Joshy Jacobs (Department of Microbiology and Immunology, Emory University School of Medicine) for his help in retroviral vector construction. Lymphocyte transformations were done by the Clinical Research Facility Service Laboratory with grant support from NIH MO1-RR00039, and some fibroblast cultures were obtained through the courtesy of the Emory Genetics Laboratory. M.M.N. was supported in part by an NIH training grant 5T32DK07734-03. We also thank Barbara Summers for her technical assistance. A preliminary report of this work was presented at the Annual Meeting of the American Society of Human Genetics, Philadelphia, October 2000.

Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

GenBank, http://www.ncbi.nlm.nih.gov/Genbank/ (for E1α [accession number Z14093], E1β [accession number M55575, and E2 [accession number X66785])

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for MSUD types Ia [MIM 248600], Ib [MIM 248611] and II [MIM 248610])

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