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ORIGINAL ARTICLE

Description of the mutations in 15 subjects with variant forms of maple syrup urine disease

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Summary
Background: In maple syrup urine disease (MSUD), disease-causing mutations can affect the *BCKDHA*, *BCKDHB* or *DBT* genes encoding for the E1α, E1β and E2 subunits of the multienzyme branched-chain 2-keto acid dehydrogenase (BCKD) complex. *Aim:* The aim of this study was to screen DNA samples of 15 subjects with distinct well-characterized variant MSUD phenotypes for mutations in the three genes in order to demonstrate a potential correlation between specific nucleotide changes and particular variant phenotypes. *Methods:* The exonic coding sequences of all three genes were studied using genomic DNA and cellular RNA derived from peripheral blood leukocytes. *Results:* In 37% of the cases (total 30 alleles), disease-causing mutations were located in the *BCKDHA*, in 46% in the *BCKDHB,* and in 13% in the *DBT* gene. Novel mutations occurring homozygously were p.Ala328Thr in the *BCKDHA* gene and p.Gly249_Lys257del in the *DBT* gene. Both are associated with a mild MSUD variant. The same holds true for the novel mutations p.Pro200Ala in *BCKDHB* and p.Phe307Ser in *DBT* which were identified in heterozygous fashion. Among the known mutant alleles, p.Gly278Ser in the *BCKDHB* gene was relatively frequent and also associated with a mild MSUD variant. *Conclusion:* The results of this study indicate that genotyping may be predictive of clinical severity of variant MSUD phenotypes and might be of prognostic value particularly in subjects with variant MSUD identified in newborn screening in whom early treatment fortunately slows the natural course of the disease.

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Abbreviations

BCAA	branched-chain amino acid
BCKD	branched-chain 2-keto acid dehydrogenase
BCKDHA	branched-chain 2-keto acid dehydrogenase E1, α-polypeptide
BCKDHB	branched-chain 2-keto acid dehydrogenase E1, β-polypeptide
DBT	dihydrolipoamide branched-chain transacylase
MSUD	maple syrup urine disease

Introduction

Maple syrup urine disease (MSUD, OMIM 248600) is caused by defective activity of the branched-chain 2-keto acid dehydrogenase (BCKD) complex. Owing to the autosomal recessively inherited metabolic block the branched-chain amino acids leucine, valine and isoleucine and the corresponding branched-chain 2-keto acids accumulate. The BCKD is a multienzyme complex composed of a multimeric dihydrolipoamide transacylase (E2) core to which multiple copies of BCKD decarboxylase (E1) and dihydrolipoamide dehydrogenase (E3) as well as two regulatory enzymes, BCKD kinase and BCKD phosphatase, are bound (Pettit et al 1978; Reed et al 1985). The E1 component exists as a heterotetramer composed of two E1α and two E1β subunits. The genomic changes that impair BCKD activity can occur in any of the catalytic components of the complex, but both alleles at a single gene locus must harbour nucleotide changes (Aevarsson et al 2000; Chuang and Shih 2001; Nellis and Danner 2001; Rodriguez-Pombo et al 2006). Based on the affected loci of the BCKD complex, three molecular MSUD genotypes are known so far: subtype Ia for mutations affecting the E1α (*BCKDHA*) gene, subtype Ib for mutations affecting the E1β (*BCKDHB*) gene and subtype II for mutations affecting the E2 (*DBT*) gene.

About 75% of MSUD patients have the severe classic form (<2% of control enzyme activity) with neonatal onset of encephalopathy and coma. About 25% of patients suffer from variant forms (with a continuum of residual BCKD activity from 2% to 40%) with later onset or absence of cerebral symptoms (Chuang and Shih 2001). Based on the clinical presentation and biochemical response to thiamine administration, variants can be divided into more severe so-called intermediate and milder so-called intermittent and thiamine-responsive forms (Chuang and Shih 2001) as well as an asymptomatic phenotype which can be identified by newborn screening (Simon et al 2006).

In the present study we analysed DNA and RNA samples of 15 subjects with distinct well-characterized more severe intermediate, and milder intermittent or asymptomatic variant MSUD phenotypes for mutations in the *BCKDHA*, *BCKDHB* and *DBT* genes of the BCKD complex in order to demonstrate a potential correlation between specific nucleotide changes and particular variant phenotypes.

Subjects, materials and methods

Fifteen individuals suffering from variant MSUD of different severity who attended various paediatric metabolic departments in Germany, Austria and Switzerland were enrolled in the study. Four patients were of Turkish origin with a consanguineous pedigree. Based on information on clinical course and treatment, laboratory data, and degree of enzyme deficiency, the individuals were assigned to one of two variant phenotypes: a more severe intermediate variant, and a milder intermittent or asymptomatic variant (Simon et al 2006). Twelve of fifteen individuals were—with identical code numbers (1, 3–7, 9–12, 14, 16)—included in a recent description of a total of 16 individuals with different forms of variant MSUD (Simon et al 2006). Patients with code numbers 30–32 are described for the first time. Patient 30 had an intermittent course of disease with two reported encephalopathic episodes at 3 and 7 years of age. MSUD was diagnosed at 13 years of age during acute metabolic decompensation (plasma leucine concentration 1.5 mmol/L) leading to lethal brain herniation. Patient 31 was identified by tandem mass spectrometry (MS/MS) newborn screening and is treated with a BCAA-reduced diet. At the age of 26 months he showed a mild metabolic decompensation during febrile illness, with a maximum plasma leucine level of 1075 μmol/L. Patient 32 was identified by MS/MS-based newborn screening and was treated with a BCAA-reduced diet. At the age of 15 months the diet was interrupted and blood leucine levels increased to about 800 μmol/L. Thus, patients 30–32 can be classified as having relatively mild MSUD variants.

Informed consent for the analyses was obtained from a parent/legal guardian of the patients. The Heinrich-Heine University Insitutional Review Board approved the study. For assessment of the pathogenicity of the novel mutations, 96 *BCKDHA*, 70 *BCKDHB* and 108 *DBT* control alleles of a German and Turkish population were studied.

Genomic DNA and total cellular RNA were extracted from peripheral blood leukocytes. Mutation analysis was performed by direct sequencing of PCR fragments obtained after amplification of the exonic and flanking intron region coding sequences of the three genes—*BCKDHA* with 9 exons, *BCKDHB* and *DBT* with 11 exons each. Primers to amplify the genomic DNA samples were designed according to GenBank sequences. All primer sequences are

available on request. Direct cycle sequencing of all PCR fragments was performed with BigDye Terminator v 3.1 mix (Applied Biosystems, Foster City, CA. USA) and analysed by capillary electrophoresis on an ABI prism 310 Genetic Analyzer (Applied Biosystems).

Analysed sequences were compared with the cDNA and genomic DNA sequences in GenBank accession numbers NM_000709 (*BCKDHA* gene, contig NT_011109), NM_000056 (*BCKDHB* gene, contig NT_007299) and NM_001918 (*DBT* gene, contig NT_028050). The mutation nomenclature used follows the recommendation of the Human Genome Variation Society (http://www.hgvs.org/mutnomen). cDNA numbering commences from the ATG start codon, where +1 is the A of the ATG translation initiation codon.

Classification of MSUD variants as severe or mild

On the basis of the clinical presentation, MSUD variants are divided into more severe, so-called intermediate, and milder, so-called intermittent or asymptomatic, phenotypes. Subjects with a more severe variant have markedly increased BCAA levels and neurological impairment, but do not have catastrophic illness with coma in the neonatal period. Without treatment the markedly increased plasma levels of BCAA (>1 mmol/L) are persistently present. In order to prevent developmental delay and neurological impairment a strict leucine-balanced diet is required. Individuals belonging to the milder category show normal early development without neurological signs. They are at risk for acute metabolic decompensation during stressful situations. While patients are asymptomatic, the plasma BCAA levels are normal or only slightly elevated. Despite having the mild form, patients can die during acute episodes owing to brain herniation if not adequately treated. They may benefit from mild dietary protein restriction (Chuang and Shih 2001; Simon et al 2006).

Results

The mutations detected by sequencing the *BCKDHA*, *BCKDHB*, and *DBT* genes from 15 clinically

Table 1 Variations detected in the *BCKDHA* gene and clinical and biochemical data of subjects with variant MSUD (subtype Ia)

Identity	Clinical phenotype of variant	BCKDH activity (% of normal)	Exon	Mutation at nucleotide level	Mutation at protein level	Estimated effect	Reference of published mutations
Patient 6	Severe (intermediate)	2.5	4	c.475C>T	p.Arg159Trp	Classic	Nobukuni et al (1993) (as R114W)
			?	?	?	–	
Patient 16[a]	Severe (intermediate)	n.d.	6	c.757G>A	p.Ala253Thr	Severe variant	Nobukuni et al (1993) (as A209T)
			6	c.757G>A	p.Ala253Thr	Severe variant	Nobukuni et al (1993) (as A209T)
Patient 1[ab]	Severe (intermediate)	2–3	7	c.868G>A	p.Gly290Arg	Severe variant	Chuang et al (1995) (as G245R)
			7	c.868G>A	p.Gly290Arg	Severe variant	Chuang et al (1995) (as G245R)
Patient 32[a]	Mild (NBS – mild course)	n.d.	7	c.919G>A	p.Arg297His	Mild variant	Chuang and Shih (2001) (as R252H)
			7	c.919G>A	p.Arg297His	Mild variant	Chuang and Shih (2001) (as R252H)
Patient 7[a]	Mild (asymptomatic)	20	7	c.982G>A	p.Ala328Thr	Mild variant	
			7	c.982G>A	p.Ala328Thr	Mild variant	
Patient 3	Severe (intermediate)	3–4	9	c.1234G>A	p.Val412Met	Severe variant	Henneke et al (2003)
			9	c.1234G>A	p.Val412Met	Severe variant	Henneke et al (2003)

n.d., not determined. NBS, newborn screening by tandem mass spectrometry.
[a] Turkish origin, consanguineous pedegree.
[b] Patient described by Henneke et al (2003).

Table 2 Variations detected in the *BCKDHB* gene and clinical and biochemical data of subjects with variant MSUD (subtype Ib)

Identity	Clinical phenotype of variant	BCKDH activity (% of normal)	Exon	Mutation at nucleotide level	Mutation at protein level	Estimated effect	Reference of published mutations
Patient 31	Mild (NBS – mild course)	n.d.	3	c.331C>T	p.Arg111X	Classic	
			5	c.598C>G	p.Pro200Ala	Mild variant	
Patient 4	Mild (asymptomatic)	19–25	4	c.368C>T	p.Pro123Leu	?	
			7	c.832G>A	p.Gly278Ser	Mild variant	Edelmann et al (2001) (as G228S)
Patient 12	Mild (intermittent)	20	4	c.389T>G	p.Val130Gly	?	
			6	c.724T>C	p.Tyr244His	?	
Patient 10[a]	Mild (intermittent)	7–9	5	c.595_596delAG	p.Pro200X	Classic	Hennecke et al (2003)
			7	c.832G>A	p.Gly278Ser	Mild variant	Edelmann et al (2001) (as G228S)
Patient 11	Mild (intermittent)	n.d.	5	c.502C>T	p.Arg168Cys	?	
			8	c.848T>C	p.Val283Ala	?	
Patient 5	Mild (asymptomatic)	17	7	c.832G>A	p.Gly278 Ser	Mild variant	Edelmann et al (2001) (as G228S)
			7	c.832G>A	p.Gly278 Ser	Mild variant	Edelmann et al (2001)
Patient 14	Mild (intermittent)	n.d.	7	c.832G>A	p.Gly278Ser	Mild variant	Edelmann et al (2001) (as G228S)
			7	c.808_821del	p.Ser270_ Leu274del	?	

[a] Patient described by Henneke et al. (2003).
n.d., not determined. NBS, newborn screening by tandem mas spectrometry.

well-characterized subjects with variant MSUD of different severity are shown in Tables 1–3. The tables also provide information on the precise clinical phenotype of variant MSUD, the residual BCKD activity in cultured fibroblasts and the estimated effect of a given mutation on the clinical phenotype. In 37% (6 subjects) of a total of 30 alleles, the supposed disease-causing mutations were located in the *BCKDHA* gene in 46% (7 subjects) in the *BCKDHB* gene and in 13% (2 subjects) in the *DBT* gene. In all cases in which parental DNA was available Mendelian inheritance was confirmed.

Mutations in the BCKDHA gene (Table 1)

All allelic variants detected in the *BCKDHA* gene were missense changes. c.475C>T (p.Arg159Trp), c.757G>A (p.Ala253Thr), c.868G>A (p.Gly290Arg), c.919G>A (p.Arg297His) and c.1234G>A (p.Val412-Met) were already known from the literature, whereas c.982G>A (p.Ala328Thr) was a novel mutation. In addition, we identified the nucleotide sequence variation c.87C>A (p.Pro39His) (registered as SNP rs 34589432) in five patients, and the nucleotide sequence variations c.34C>A (p.Arg12Arg) (SNP rs 34541442)

Table 3 Variations detected in the *DBT* gene and clinical and biochemical data of subjects with variant MSUD (subtype II)

Identity	Clinical phenotype of variant	BCKDH activity (% of normal)	Exon	Mutation at nucleotide level	Mutation at protein level	Estimated effect	Reference of published mutations
Patient 30	Mild (intermittent)	n.d.	2	c.75_76delAT	p.Cys26Trp fsX1	Classic	Fisher et al (1993) (as c.89_90delAT)
			7	c.920T>C	p.Phe307Ser	Mild variant	
Patient 9	Mild (intermittent)	4	6	c.747_773del	p.Gly249_Lys257del	Mild variant	
			6	c.747_773del	p.Gly249_Lys257del	Mild variant	

n.d., not determined.

in two alleles, c.972C>T (p.Phe324Phe) (SNP rs 284652) in seven alleles, and c.1222A>G (p.Leu407-Leu) (SNP rs 4647) in nine alleles of all subjects.

Mutations in the BCKDHB gene (Table 2)

In the *BCKDHB* gene we identified eight nucleotide substitutions and two deletions. Among the ten mutations only c.832G>A (p.Gly278Ser) had been previously described and the genotype of patient 10 was previously reported by our group (Henneke et al 2003). Novel mutations were c.331C>T (p.Arg111X), c.368C>T (p.Pro123Leu), c.389T>G (p.Val130Gly), c.502C>T (p.Arg168Cys), c.598C>G (p.Pro200Ala), c.724T>C (p.Tyr244His), c.848T>C (p.Val283Ala) and c. 808_821del (p.Ser270_Leu274del).

Mutations in the DBT gene (Table 3)

In the DBT gene we identified two novel mutant alleles, the c.747_773del (p.Gly249_Lys257del) allele in homozygous fashion and the c.920T>C (p.Phe307Ser) allele in heterozygous fashion. One subject harboured the previously described c.75_76delAT (p.Cys26Trp fsX1) mutation. In addition, we identified the nucleotide sequence variation c.1150G>A (p.Gly384Ser) (registered as SNP rs 12021720) in two patients.

Pathogenicity of the novel mutations was assessed by discarding their presence in 96 *BCKDHA*, 70 *BCKDHB* and 108 *DBT* control alleles. None of the novel mutations was registered as a non-synonymous coding single-nucleotide polymorphism. Most variations described here affect highly conserved residues between the human E1 or E2 component and their homologous proteins as compared in the nucleotide databases from bacterial (*Pseudomonas putida*) and animal (*Bos taurus*, *Rattus norvegicus*, *Gallus gallus*) genomes (Aevarsson et al 2000; Ono et al 2001), strengthening their impact on the structure/function of the proteins. In addition, the disease-causing effect was assumed when the alteration led to a premature termination codon.

Discussion

The previously described mutations in the *BCKDHA* gene, p.Arg159Trp, p.Ala253Thr, p.Gly290Arg and p.Val412Met, have been reported to be associated with neonatal classic or severe variant MSUD. Three of our patients with a severe variant MSUD phenotype—that is, clear cerebral symptoms, but lacking coma in the neonatal period—carried either p.Ala253Thr, p.Gly290Arg or p.Val412Met in homozygous fashion. At least homozygous p.Gly290Arg and p.Val412Met were associated with very low (2–4%) residual BCKD activity in cultured fibroblasts. Thus, for the homoallelic p.Gly290Arg genotype the prediction of a severe variant MSUD phenotype with neonatal manifestation (Chuang et al 1995; Rodriguez-Pombo et al 2006) is verified. The same holds true for the previously described p.Ala253Thr mutation (Nobukuni et al 1993) and the p.Val412Met mutation (Henneke et al 2003) in the *BCKDHA* gene. In patient 6, who presented with the clinical course of a severe MSUD variant, we identified only one variation in the *BCKDHA* gene. In this patient the allele associated with variant MSUD remains obscure: most probably it is not p.Arg159Trp because this mutation is associated with classic MSUD (Nobukuni et al 1993).

Apart from these mutations associated with a severe variant course, we identified two allelic variants in the *BCKDHA* gene associated with a mild clinical course. Both allelic variants were present in homozygous fashion: the previously described p.Arg297His (Chuang and Shih 2001) in a relatively mildly affected boy who was identified by MS/MS-based newborn screening and the novel mutation p.Ala328Thr in a hitherto asymptomatic 9-year-old boy. The latter homoallelic genotype was associated with high residual BCKD activity (20%) in fibroblasts.

Whereas the known *BCKDHA* mutations affect highly conserved residues of the E1α subunit and either cofactor binding, hydrophobic cores or subunit association (Aevarsson et al 2000; Henneke et al 2003), the novel p.Ala328Thr mutation does not affect a highly conserved residue of the E1α subunit (Aevarsson et al 2000) and appears to have only small effect on protein structure and stability.

Most of our patients with milder (intermittent or asymptomatic) MSUD variants and higher residual enzyme activities had mutations in the *BCKDHB* gene. Among these patients only patient 5 was homozygous for a single mutation while six patients were compound heterozygotes for two different mutations in the *BCKDHB* gene.

From the genotypes of patients 10 and 31 it can be concluded that p.Gly278Ser and p.Pro200Ala must be associated with variant MSUD, because in the other alleles (p.Pro200X and p.Arg111X) a nonsense codon was introduced by the mutation, causing a quasi-functionally hemizygous condition. Among the novel mutations p.Pro123Leu, p.Val130Gly, p.Arg168Cys, p.Tyr244His, p.Val283Ala and p.Ser270_Leu274del occurring in compound heterozygous fashion, the assignment of an individual mutation to the variant

MSUD phenotype is not possible. In total, four subjects carried p.Gly278Ser: one subject in a homozygous fashion whereas three were compound heterozygotes for this mutation and other different mutations affecting the *BCKDHB* gene. Obviously, p.Gly278Ser was associated with relatively high residual BCKDH activity (>10%) and a mild variant phenotype with an intermittent course of the disease or even with the patient remaining asymptomatic. Originally, this mutation was reported to be associated with a variant MSUD by Edelmann and colleagues (2001).

All novel mutations affect highly conserved residues of the E1β subunit, and according to the crystal structure of the human E1 component all affected residues are located in essential secondary structure elements. The mutations p.Pro123Leu, p.Val130Gly and p.Tyr244His might affect cofactor binding, p.Val283Ala and p.Pro200Ala subunit association, and p.Arg168Cys stability of E1β subunit by conformational changes, whereas p.Ser270_Leu274del might cause loss of important functional areas of the E1β subunit by the generation of an incorrect amino acid sequence.

In the patient group with subtype II MSUD, a novel c.747_773del (p.Gly249_Lys257del) mutant allele occurred homozygously in a female with a mild (intermittent) MSUD variant. For this allele the genomic point mutation corresponding to cDNA position c.747C>T generates a new splicing signal, which finally results in a shortening of the amino acid sequence of exon 6 by nine amino acids. Accordingly, in the sequencing of mRNA we identified a loss of 27 bp. Both the mutated c.747C>T and the preceding c.746G are cut out by splicing of this fragment. As a consequence, at DNA level the original nucleotide sequence ends with c.745"G" and is followed by the nucleotides of exon 7. Since the nucleotide sequence of exon 7 starts with "GC", a "GGC" triplet is formed coding for glycine. Obviously the in-frame deletion leads to a loss of nine amino acids in a nonessential part of the E2 subunit. From the genotype of patient 30 it can be concluded that the novel p.Phe307Ser mutation must be associated with variant MSUD, because in the other allele c.75_76delAT (p.Cys26Trp fsX1) a nonsense codon was introduced by the mutation causing a quasi-functionally hemizygous condition.

In conclusion, in our cohort the more severe enzyme and clinical phenotypes of variant MSUD were mainly associated with specific genotypes in the *BCKDHA* gene whereas the milder enzyme and clinical phenotypes were associated with specific genotypes in the *BCKDHB* and *DBT* genes. The results of this study support the conclusion that genotyping may be predictive of metabolic and clinical phenotype and might be of prognostic value particularly in subjects with variant MSUD identified in newborn screening in whom early treatment fortunately slows the natural course of the disease.

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