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Analysis of gene mutations among South Indian patients with maple syrup urine disease: Identification of four novel mutations

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Maple syrup urine disease (MSUD) is predominantly caused by mutations in the BCKDHA, BCKDHB and DBT genes, which encode for the E1α, E1β and E2 subunits of the branched-chain α-keto acid dehydrogenase complex, respectively. Because disease causing mutations play a major role in the development of the disease, prenatal diagnosis at gestational level may have significance in making decisions by parents. Thus, this study was aimed to screen South Indian MSUD patients for mutations and assess the genotype-phenotype correlation. Thirteen patients diagnosed with MSUD by conventional biochemical screening such as urine analysis by DNPH test, thin layer chromatography for amino acids and blood amino acid quantification by HPLC were selected for mutation analysis. The entire coding regions of the BCKDHA, BCKDHB and DBT genes were analyzed for mutations by PCR-based direct DNA sequencing. BCKDHA and BCKDHB mutations were seen in 43% of the total ten patients, while disease-causing DBT gene mutation was observed only in 14%. Three patients displayed no mutations. Novel mutations were c.130C>T in BCKDHA gene, c. 599C>T and c.121_122delAC in BCKDHB gene and c.190G>A in DBT gene. Notably, patients harbouring these mutations were non-responsive to thiamine supplementation and other treatment regimens and might have a worse prognosis as compared to the patients not having such mutations. Thus, identification of these mutations may have a crucial role in the treatment as well as understanding the molecular mechanisms in MSUD.

Keywords: Maple syrup urine disease, BCKD complex, BCKDHA, BCKDHB, DBT, Mutation analysis

Maple syrup urine disease (MSUD, OMIM 248600) is caused by defective activity of the branched-chain α-keto acid dehydrogenase (BCKD) complex within the matrix of the mitochondria. Owing to the recessive nature of this autosomaly inherited deficiency of BCKD, the branched-chain amino acids leucine, valine, isoleucine and their branched-chain α-keto acids accumulate in cells and body fluids[1] (Fig. 1). This leads to abnormalities in neurological development in untreated infants[2].

MSUD has an incidence of 1 in 150,000 live-births in the general population and 1/176 for the Mennonite

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Abbreviations: BCCA, branched chain amino acids; BCKD, branched chain α - ketoacid dehydrogenase; BCKDHA, branched-chain α-keto acid dehydrogenase E1, α-polypeptide; BCKDHB, branched-chain α-keto acid dehydrogenase E1, β-polypeptide; DBT, dihydrolipoamide branched-chain transacylase; DNPH test, dinitrophenyl hydrazine test; HGMD, human gene mutation database; HPLC, high performance liquid chromatography; MSUD, maple syrup urine disease; OMIM, online mendelian inheritance in man; PCR, polymerase chain reaction.

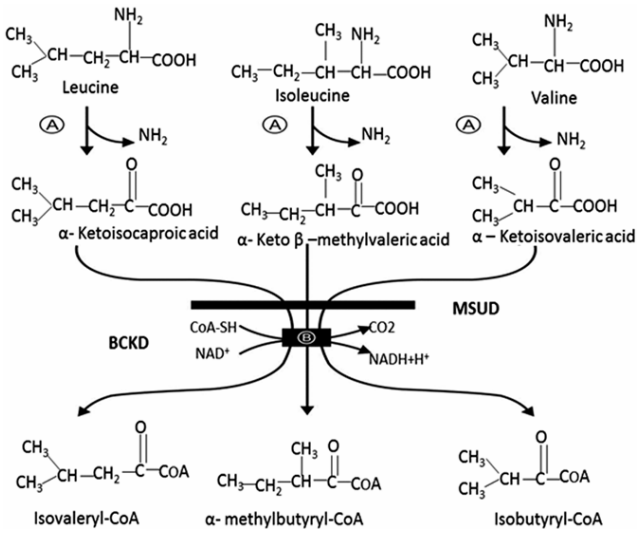

Fig. 1—Catabolic pathways for the branched chain amino acids (BCAA) leucine, isoleucine and valine [(A) The first reaction is catalyzed by BCCA aminotransferases; and (B) The second reaction is oxidative decarboxylation, catalyzed by a single branched chain α ketoacid dehydrogenase complex (BCKD). The metabolic block at the second reaction results in MSUD]

population[3]. Human gene mutation database shows that more than 100 mutations have been identified, so far (HGMD; http://www.biobase-international.com/product/hgmd). Mutations in any of the three different genes — BCKDHA, BCKDHB and DBT, encoding the E1α, E1β, and E2 catalytic components of the branched-chain α-ketoacid dehydrogenase complex can cause MSUD[4]. Disease severity ranges from the classic to the mildest variant types based on the genotypes[5].

The BCKD is a multi-enzyme complex composed of a multi-meric 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[6,7]. 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 the alleles at a single genetic locus must harbor nucleotide changes[8-11].

Based on the affected loci of the BCKD complex, three molecular MSUD genotypes are known so far: subtypes Ia, Ib and II for mutations affecting the BCKDHA, BCKDHB and DBT genes, respectively. 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[9]. Based on the clinical presentation and biochemical response to thiamine administration, MSUD variants can be divided into more severe, so-called intermediate and milder, so-called intermittent and thiamine-responsive forms[9], as well as an asymptomatic phenotype which can be identified by new-born screening[12].

The importance of molecular studies is emphasized because they facilitate diagnosis, treatment and allow an adequate genetic counselling[13]. In MSUD, mutations are more common in BCKDHA and BCKDHB genes than in DBT gene[10]. Since the E3 component is also a part of other mitochondrial enzymes, clinical features due to mutations in DBT are different from classical MSUD. Individuals with MSUD are always homozygous or compound heterozygous for mutations in the same subunit gene. Most MSUD patients are compound heterozygotes for rare sequence variants. Depending on the interaction between the two mutant alleles with respect to compound heterozygotes, the clinical phenotype may vary. No single mutation or gene accounts for a significant proportion of affected alleles, except in genetic isolates[14].

In the present study, we have screened DNA samples of thirteen patients with MSUD, including diverse clinical phenotypes and ethnic backgrounds 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. In addition, we report four novel mutations in BCKDHA BCKDHB and DBT genes in four of these patients.

Materials and Methods

Patients

All the patients included in this study were treated at the Amrita Institute of Medical Sciences and Research Centre, Cochin, India. Thirteen patients with MSUD, each from a different family, were recruited for the study. These families were found to be not related to each other as per the records. The families were from different parts of states located in South India, including North Kerala, South Karnataka and adjoining areas of Tamil Nadu. Average age at diagnosis was 24 months and mean age at presentation was 7 months. Informed consent for the analysis of samples was obtained from parents or guardians of the patients. Amrita Viswavidyapeetham University Institutional Ethics Review Board approved the study.

Diagnosis

A total of 420 high-risk patients were screened for MSUD initially. Thirteen patients were diagnosed with MSUD based on clinical features and biochemical tests, such as urine screening by DNPH test[15] and thin layer chromatography for amino acids[16]. Diagnosis was confirmed by blood amino acid quantification using HPLC[17,18]. The classical burnt sugar odour in urine was noted in majority of patients (85%). Response to dietary thiamine (vitamin B1) therapy was monitored in all patients, as follows: vitamin B1 (50–300 mg/day) was administered for at least three weeks in combination with a low protein diet; levels of leucine and valine in the blood as well as levels of branched-chain α-keto acids in urine were analyzed[19].

PCR and DNA sequencing

Isolation of DNA from blood samples

Blood samples were collected from respective patients with confirmed MSUD and stored in EDTA solution at –70°C. Genomic DNA was isolated from

200 µl of each blood sample using a commercial DNA isolation kit (Qiagen, USA). For polymerase chain reaction (PCR), the DNA was diluted to 25 ng/µl and 4 µl was used in a 25 µl PCR reaction.

Preparation of PCR products

Genomic DNA was extracted from peripheral blood leukocytes. Genetic analysis was performed by DNA 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 with 10 exons and DBT with 11 exons each. Primers to amplify the genomic DNA samples were designed by using primer 3.0 software, according to GenBank sequences. A total of 40, 42 and 45 PCR reactions were carried out to screen for BCKDHA, BCKDHB and DBT genes, respectively.

The primers for each exon were located at least 50 bp away from exon-intron boundaries. Non-coding exons were excluded from the study. The composition of the 10X buffer used was 100 mM Tris-HCl pH 9.0, 15 mM $MgCl_2$, 500 mM KCl, 0.1% gelatin. PCR was performed in 25 µl solutions containing 100 ng of genomic DNA, 1X PCR buffer, 100 µM dNTPs (Gibco BRL), 10 pmol of each primer (Sigma) and 0.5 U of Taq DNA polymerase (Merck Millipore). Reactions were carried out in a thermal cycler (Biorad) as follows: 95°C for 3 min, 40 cycles (95°C for 30 s, 58°C for 1 min, and 72°C for 30 s) and 72°C for 10 min. Depending on the exons, the annealing temperature varied from 58°C to 69°C.

Direct sequencing of PCR products

All the patient DNA samples were amplified and the amplicons were purified using PCR purification kit (Qiagen) and subjected to automated DNA sequencing (313 XL genetic analyzer; Applied Biosystems) using the manufacturer's suggested protocols. Sense and antisense strand sequencing were done to confirm all mutations.

Results

In 420 high-risk cases, thirteen cases of MSUD were biochemically diagnosed and subjected to molecular genetic analysis. Five of the patients died during the last follow-up and were of classical form. There was a family history in 5/13 (38%) cases and first degree consanguinity in 4/13 (30%) of cases. The genomic DNA from thirteen independent patients of MSUDs were subjected to PCR-based direct DNA sequencing of the entire coding regions of the BCKDHA, BCKDHB and DBT genes. We found mutations in 10/13 (77%) of patients. The total DNA from 100 healthy unrelated normal controls was tested to confirm the absence of the identified mutations in the normal population. The web server Hansa (hansa.cdfd.org.in:8080) predicted missense mutations as "disease"[20]. Six mutations were identified in the BCKDHA and BCKDHB genes each. Two mutations were identified in DBT gene. In these mutations, four mutations were novel. The mutation nomenclature used followed the recommendation of the Human Genome Variation Society (http://www.hgvs.org/mutnomen)[21].

Mutations in the BCKDHA gene

All allelic variants detected in the BCKDHA gene were missense changes, except the novel mutation detected in exon 2 (Table 1). Mutations c.1036C>T (p.Arg346Cys), c.308T>C (p.Leu103Pro), c.475C>T (p.Arg159Trp), c.1312T>C (p.Tyr438His) and c.982G>A (p.Ala328Thr) were already known from the literature[1,22,23]. The novel mutation c.130C>T (p.Gln44X) detected was a nonsense mutation. Patient OA-08 was compound heterozygous, patient OA-27 was heterozygous and all other patients were homozygous.

Mutations in the BCKDHB and DBT genes

In the BCKDHB gene, we identified three missense mutations, two nonsense mutations and one deletion (Table 2). Among the six mutations, c.853C>T

Table 1—Variations detected in the BCKDHA gene and genotype phenotype correlation of subjects with MSUD

Patient	Age	Mutation		Position	Mutation type	Clinical phenotype
		Nucleotide level	Protein level			
OA-08	F/6m	c.1036C>T	p.Arg346Cys	Exon 8	Missense	Classic
		c.130C>T	**p.Gln44X**	Exon 2	Nonsense	
OA-27	M/1y	c.475C>T	p.Arg159Trp	Exon 4	Missense	Classic
OA-33	F/8m	c.1312T>C	p.Tyr438His	Exon 9	Missense	Mild variant
OA-03	M/6m	c.982G>A	p.Ala328Thr	Exon 7	Missense	Mild asymptomatic
OA-05	M/7m	c.308T>C	p.Leu103Pro	Exon 3	Missense	Classic

Reference sequence: GenBank accession number BCKDHA- NM_000709.3, Ensemble Gene ID BCKDHA-ENSG00000248098. Novel mutations are shown in bold face. cDNA and amino acid nomenclature considers "A" of translation initiation codon (ATG) as the first nucleotide and ATG/methionine as the first codon/amino acid, respectively.

Table 2—Variations detected in BCKDHB & DBT genes and genotype phenotype correlations in subjects with MSUD

Patient	Age	Mutation		Position	Mutation type	Clinical phenotype
		Nucleotide level	Protein level			
BCKDHB						
OA-14	M/6m	c.853C>T	p.Arg285X	Exon 8	Nonsense	Mild asymptomatic
OA-19	M/7m	c.403G>A	p.Gly135Arg	Exon 4	Missense	Mild variant
		c.599C>T	**p.Pro181Leu**	Exon 5	Missense	
OA-38	M/8m	c.475C>A	p.Gln159lys	Exon4	Missense	Classic
OA-29	F/4m	c.970C>T	p.Arg324X	Exon 9	Nonsense	Mild variant
		c.121_122delAC		Exon 1		
DBT						
OA-20	F/10m	c.827T>G	p.Phe276Cys	Exon 7	Missense	Classic
		c.190G>A	**p.Val64Ile**	Exon 3	Missense	

Reference sequences: GenBank accession numbers (BCKDHB- NM_000056.3; DBT- NM_001918.3) Ensemble Gene ID (BCKDHB-ENSG00000083123; DBT -ENSG00000137992). Novel mutations are shown in bold face. cDNA and amino acid nomenclature considers "A" of translation initiation codon (ATG) as the first nucleotide and ATG/methionine as the first codon/amino acid, respectively.

(p.Arg285X), c.403G>A (p.Gly135Arg), c.475C>A (p.Gln159Lys) and c.970C>T (p.Arg324X) had been previously described[22,24]. Novel mutations were c.599C>T (p.Pro181Leu) and c.121_122delAC. Patients OA-19 and OA-29 were compound heterozygous and patient OA-14 and OA-38 were homozygous. In DBT gene, c.827T>G (p.Phe276Cys) was previously reported[24] and c.190G>A (p.Val64Ile) was a novel mutation. The patient was compound heterozygous and this was the first DBT gene mutation reported from India.

Pathogenicity of the novel mutations was assessed by discarding their presence in 100 BCKDHA, BCKDHB and DBT control alleles. Most variations described here affect highly conserved residues in the human E1 or E2 components[8,25], strengthening their impact on the structure or function of the proteins. In addition, the disease-causing effect was assumed when the alteration led to a premature termination codon.

Discussion

In this study, 13 MSUD patients were analysed for BCKDHA, BCKDHB and DBT gene mutations by PCR-based direct DNA sequencing. Patients were diagnosed with MSUD based on clinical features and biochemical tests. The clinical features were excessive irritability, abnormal posturing since birth and delayed developmental milestones[18]. Preliminary diagnosis was done by biochemical screening, such as urine analysis by DNPH test[15] and thin layer chromatography for amino acids[16]. Diagnosis was confirmed by blood amino acid quantification using HPLC[17,18].

The contribution of BCKDHA, BCKDHB and DBT gene mutations to MSUD in India remains largely unexplored. The only reported molecular study from South India reveals that BCKDHA and BCKDHB could be the major genes causing MSUD in Indian population[22]. Our study also detected 12/14 (86%) mutations in BCKDHA and BCKDHB genes. Equal frequency of mutations in the two genes is also reported earlier[1,10]. In addition, we also identified 4/14 (29%) novel disease causing mutation in BCKDHA, BCKDHB and DBT genes. This is first molecular genetic analysis on entire coding region of BCKDHA, BCKDHB and DBT genes causing MSUD from South Indian population.

Some amino acid residues of BCKDHA, such as BCKDHA Arg346[11,22], BCKDHA Tyr438[10,24,26], BCKDHA Leu103[23,27] and BCKDHA Arg159[1,28] are most frequently affected in MSUD patients. The BCKDHA Tyr438His and BCKDHB Arg324X have been shown to be affected previously in Indian MSUD patients[22]. The c.308T>C (p.Leu103Pro) mutation in BCKDHA gene is reported three-times from Asian countries[23,27]. The BCKDHB c.403G>A (p.Gly135Arg) and BCKDHB c.475C>A (p.Gln159lys) have been previously reported from Germany[24].

Most of our patients with milder (intermittent or asymptomatic) MSUD had mutations in the BCKDHB gene. Five patients were homozygous and one patient was heterozygous for a single mutation, while four patients were compound heterozygotes for two different mutations in the BCKDHA, BCKDHB and DBT genes. The BCKDHB c.853C>T (p.Arg285X) and BCKDHB c.970C>T (p.Arg324X) mutations have been reported to induce the degradation of transcript[22]. The known BCKDHA mutations affect highly conserved residues of the E1α subunit and either cofactor binding, hydrophobic cores or subunit association[8,24], whereas the p.Ala328Thr mutation does

not affect a highly conserved residue of the E1α subunit[8] and appears to have only small effect on protein structure and stability. The BCKDHA c.1036C>T (p.Arg346Cys) mutation has been shown to disrupt the hydrogen bonding network destabilizing the structure of the phosphorylation loop[29].

Mutations occurring in exons 4, 5 and 6 of BCKDHB account for more than 60% of mutations listed in human gene mutation database (HGMD), while we found only one mutation in exon 5, two in exon 4 and none in exon 6. About 50% mutations of BCKDHA gene listed in HGMD were in exons 6 and 7[22], while we detected one mutation in exon 7. Thus, the majority of mutations 7/12 are located towards the N-terminal end of the E1α and E1β subunits, resulting probably in destabilization of the $\alpha^2\beta^2$ complex[8]. In the fourteen mutations identified, four mutations appeared to result in a truncated protein. They were one nonsense mutations in the BCKDHA gene, one deletion and two nonsense mutations in BCKDHB gene. Mutations BCKDHB c.853C>T, c.970C>T, BCKDHA c.130C>T appeared to code for a stop codon in the transcribed mRNA and possibly resulted in a non-functional protein product. BCKDHB c.121_122delAC appeared to alter the reading frame of the gene and resulted in a truncated protein.

In conclusion, identification of mutation spectrum in Indian population has important implications for disease management and genetic counselling. In our study, more severe clinical phenotypes of MSUD were often associated with mutations in the BCKDHA gene, whereas the milder clinical phenotypes were often associated with specific mutations in the BCKDHB gene. Genotyping might be predictive of metabolic and clinical phenotype in subjects with MSUD. Patients harbouring mutations had a worse prognosis and were usually non-responsive to thiamine supplementation and other treatment regimens. Thus, molecular diagnosis may have a role in prenatal diagnosis of high-risk pregnancies and carrier detection in high-risk family members.

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