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Molecular Genetic Analysis of MSUD From India Reveals Mutations Causing Altered Protein Truncation Affecting the C-Termini of E1α and E1β

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ABSTRACT

Maple Syrup Urine Disease is a rare metabolic disorder caused by reduced/absent activity of the branched chain α-Ketoacid dehydrogenase enzyme complex. Mutations in *BCKDHA*, *BCKDHB*, and *DBT*, that encode important subunits of the enzyme complex namely E1α, E1β, and E2, are the primary cause for the disease. We have performed the first molecular genetic analysis of MSUD from India on nine patients exhibiting classical MSUD symptoms. *BCKDHA* and *BCKDHB* mutations were identified in four and five patients, respectively including seven novel mutations namely the *BCKDHA* c.1249delC, c.1312T>C, and c.1561T>A and the *BCKDHB* c.401T>A, c.548G>A, c.964A>G, and c.1065delT. The *BCKDHB* c.970C>T (p.R324X) mutation was shown to trigger nonsense mediated decay-based degradation of the transcript. Seven of the total 11 mutations resulted in perturbations in the E1α or E1β C-termini either through altered termination or through an amino acid change; these are expected to result in disruption of E1 enzyme complex assembly. Our study has therefore revealed that *BCKDHA* and *BCKDHB* mutations might be primarily responsible for MSUD in the Indian population. J. Cell. Biochem. 113: 3122–3132, 2012.

KEY WORDS: MSUD; MUTATION; BCKDHA; BCKDHB; TRUNCATION

Maple Syrup Urine Disease (MSUD) is a rare autosomal recessive disorder caused due to malfunctioning of the branched chain α-ketoacid dehydrogenase enzyme complex (BCKD) [Chuang et al., 2006]. BCKD is responsible for the oxidative decarboxylation of branched chain ketoacids, formed due to transamination of branched chain amino acids including leucine, isoleucine, and valine [Quental et al., 2008]. MSUD is an inborn error of metabolism and can be fatal if not treated; clinical symptoms including seizures, mental retardation, and coma are caused due to accumulation of branched chain amino acids [Nellis et al., 2003; Chuang et al., 2006]. Patients are usually managed through diet control including reduced intake of branched chain amino acids [Snyderman et al., 1964].

BCKD is a large enzyme complex constituted by three catalytic components namely a multimeric core of dihydrolipoyl acyltransferase (E2) in the form of a homo 24-mer to which are bound multiple subunits of BCKD decarboxylase (E1) and the dihydrolipoamide dehydrogenase (E3) as well as two regulatory subunits namely BCKD kinase and BCKD phosphatase. The enzyme complex is located in the mitochondria and is coded by four unlinked genes.

Additional supporting information may be found in the online version of this article.

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TABLE I. *BCKDHA* and *BCKDHB* PCR Primer Sequences

Gene/exon	Primer sequence	Annealing temperature
A		
BCKDHA_E1F	CCATTTTCAGCACGGATTTT	60.0
BCKDHA_E1R	GTCTCCCACTCTTTTTCCCTTT	
BCKDHA_E2-3F	GTTATCCAAAGTGTCGCAGTGA	60.0
BCKDHA_E2-3R	AACCCTCAGAACTCTATGGAACC	
BCKDHA_E4F	CCTCTGGCAGTTCTAAGCAGTC	60.0
BCKDHA_E4R	CACTACACTTTCTGGCCTTCAG	
BCKDHA_E5F	GCTGGGCAGAGTCAGTCA	60.0
BCKDHA_E5R	AGAAGGCAGGCAAAAGAGC	
BCKDHA_E6F	AGTGTGAATGAGTGTGAGTGC	60.0
BCKDHA_E6R	AAGTGCCAGACGCCACAG	
BCKDHA_E7F	TCGTGCATGTTCCTTATCTCAGC	57.5
BCKDHA_E7R	GTCAGTGCTGTGGGGGTGCT	
BCKDHA_E8F	CATCTCCCCCTTGCCTTTAT	60.0
BCKDHA_E8R	CACAGAGCCAGGACACACAT	
BCKDHA_E9F	TAGCCTGCCCACTGCCCCATGT	56.0
BCKDHA_E9R	CCCAAACTCCAGGAAACAAA	
B		
BCKDHB_E1F	GCTGCATAGCCTGAGAATCC	58.5
BCKDHB_E1R	AATAAGCTGGGATGCAAGGA	
BCKDHB_E2F	ATTTTGCCCCATTAACAAGC	60.0
BCKDHB_E2R	GCTACCACAATTCAGGCACA	
BCKDHB_E3F	GACAGACCCTCACAACAAAAGA	52.8
BCKDHB_E3R	GCGTTGGAAATGAAAAGGAA	
BCKDHB_E4F	GACATTACTCTCATTTGCCAC	58.2
BCKDHB_E4R	GGAAGGGTAGCGGCAATACT	
BCKDHB_E5F	AGGAGATTGGAAGGGAAGGA	58.5
BCKDHB_E5R	AACTGGGCATTGGATAGCAT	
BCKDHB_E6F	AGCCCTTCTTAGCAGCGAGT	58.2
BCKDHB_E6R	GGCTAGATGAATTTTTCCCAAA	
BCKDHB_E7F	TGCACAAGTGTCACCTCAGA	50.0
BCKDHB_E7R	GAAATTAGCATCAGTAGCACCA	
BCKDHB_E8F	ACCTTCTACATGCCATCTTTGT	56.0
BCKDHB_E8R	GCCAAAGGTTTCAGGGAAAT	
BCKDHB_E9F	ACCTGTCGAAAGCGAGTTGT	56.0
BCKDHB_E9R	TCTTCTGGAATTGGCATGTG	
BCKDHB_E10F	AAAACTGGGATCATGCGAAC	52.8
BCKDHB_E10R	CGTTAATGTCAGGGGCACAT	

The E1 subunit, a thiamine pyrophosphate (TPP)-dependent decarboxylase, is a heterotetrameric complex with a subunit structure of $\alpha 2\beta 2$. Based on the affected loci, three MSUD subtypes have been proposed namely type Ia (mutated *BCKDHA* gene coding for the E1α subunit), Ib (mutated *BCKDHB* gene coding for the E1β subunit) and II (mutated *DBT* gene coding for the E2 subunit). Mutations are more common in *BCKDHA* and *BCKDHB* than in *DBT* [Nellis and Danner, 2001]. Since the E3 component is also a part of other mitochondrial enzyme complexes, clinical symptoms due to mutations in DBT are different from classical MSUD.

MSUD has been described from diverse ethnicities with an estimated frequency of 1:1,85,000 [Danner and Doering, 1998] and more than 100 mutations have been identified so far (HGMD; http://www.biobase-international.com/product/hgmd). In the current study, we have performed the first molecular genetic study of MSUD from the Indian population and identified seven novel mutations in the BCKDHA and BCKDHB genes in nine patients.

MATERIALS AND METHODS

PATIENTS

The study was approved by the institute ethics committee. Blood samples were collected from the patients, family members, and normal subjects following informed consent. All patients were from the South Indian state of Andhra Pradesh except family 9 that belonged to North India and were diagnosed based on classical symptoms and elevated plasma levels of branched chain amino acids. Detailed clinical features of each patient are given in Supplementary document S1.

MOLECULAR GENETIC ANALYSES

Genomic DNA was isolated from blood samples as per established protocols [Bashyam et al., 2004]. Mutations were identified by direct polymerase chain reaction-DNA sequencing as per standard protocols; primer sequences for each of the nine *BCKDHA* and 10 *BCKDHB* exons are given in Tables IA and IB. Each mutation was confirmed by bi-directional DNA sequencing. Fibroblast culture from skin biopsy of proband 5 and from a normal individual was established following informed consent and used to quantitate *BCKDHB* transcript levels as described in Supplementary Methods S1.

TABLE IIA. Mutations Identified in MSUD Patients

						Family status	
Family[a]	Gender/age	Mutation[b]	Location	Mutation type	Consanguinity	Mother	Father
BCKDHA							
01	F/1 year	c.1036C>T (p.R346C)	Exon 8	Missense	Present	Carrier	Carrier
02	F/8 months	**c.1249delC**	Exon 9	Deletion	NA	NA	NA
03	F/10 months	**c.1312T>C (p.Y438H)**	Exon 9	Missense	Absent	Carrier	Carrier
04	F/8 months	**c.1561T>A**	3′-UTR	3′-UTR	NA	Normal	Carrier
BCKDHB							
05	M/20days	c.853C>T (p.R285X)	Exon 8	Nonsense	Present	Carrier	Carrier
06	F/25 days	c.970C>T (p.R324X)	Exon 9	Nonsense	Present	NA	NA
07	F/1 month	c.1016C>T (p.S339L)	Exon 9	Missense	Present	Carrier	Carrier
08	F/5 days	**c.548G>A (p.R183Q);**	Exon 5;	Missense;	Present	NA	NA
		c.964A>G (p.T322A)	Exon 9	Missense			
09	M/9 days	**c.401T>A (p.I134N);**	Exon 4;	Missense;	Absent	Carrier;	Normal;
		c.1065delT	Exon 10	Deletion		Normal	Carrier

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. Reference sequences: *BCKDHA*—GenBank accession no. NM_000709.3; *BCKDHB*—GenBank accession no. NM_000056.3.
[a]Mutations in family 1, 2, 3, 5, 6, and 7 were homozygous, in family 4 was heterozygous and in family 8 and 9 were compound heterozygous.
[b]Novel mutations are shown in bold face; F, female; M, male; UTR, untranslated region; NA, not available.

TABLE IIB. Evaluation of Missense Mutations Identified in MSUD Patients

Mutation[a]	Domain	Structural explanation	Gribskov's score	Predicted mutation status by Hansa	Percent solvent accessibility (monomer-complex)[e]
BCKDHA					
p.R346C	E1_dh[b]	Destabilization of phosphorylation loop region	+5.00 to −3.00	Disease	–
p.Y438H	E1_dh[b]	Disruption of side chain-side chain H-bonds and hence α–β′ and α′–β associations	+7.00 to +2.00	Disease	25.18–4.11 α–β′/α′–β
BCKDHB					
p.I134N	Transket_pyr[c]	Destabilization of the helical H-bond and hence destabilization of helix	+4.00 to −3.00	Disease	2.10–0.00 α–β/α′–β′
p.R183Q	Transket_pyr[c]	Loss of salt bridges; destabilization of beta sheet	+5.00–1.00	Disease	–
p.T322A	Transketolase_C[d]	Loss of proton donor for H-bonds involving neighboring polar side chain; destabilization of β subunit	+5.00–0.00	Disease	–
p.S339L	Transketolase_C[d]	Loss of proton donor for H-bond involving neighboring polar side chain; disruption of E1 β dimerization	3.79 to −1.93	Disease	11.92–0.54 β–β′

[a]Novel mutations are shown in bold face.
[b]Dehydrogenase E1 component.
[c]Transketolase, pyrimidine binding domain.
[d]Transketolase, C-terminal domain.
[e]Refer to Supplementary Table S2.

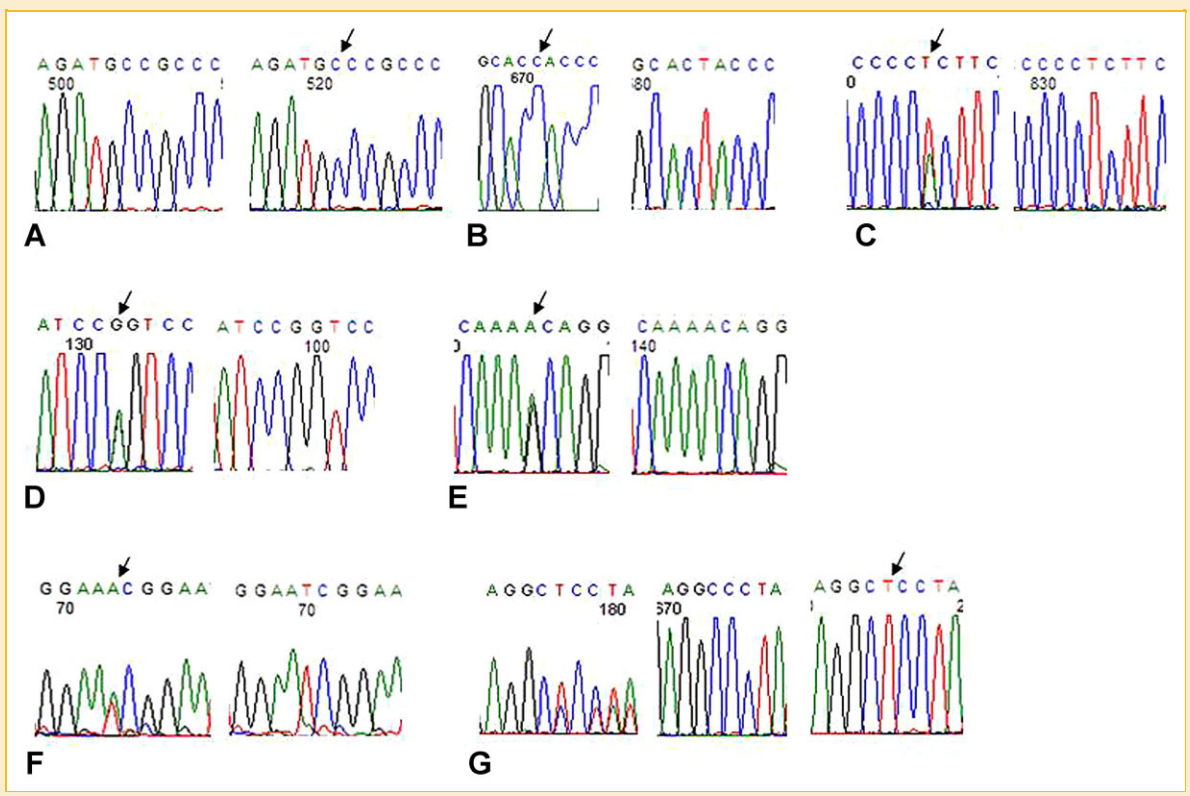
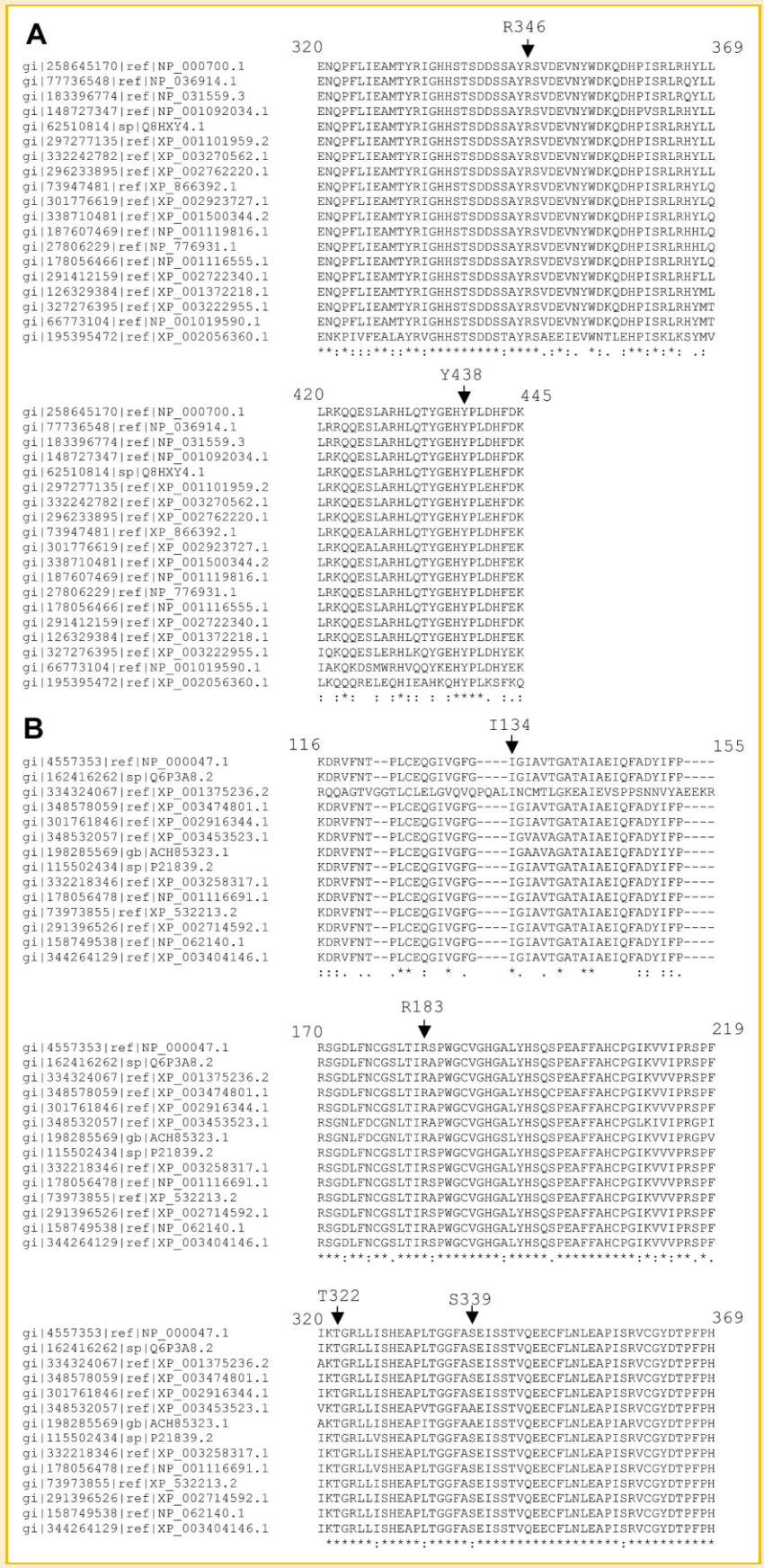


Fig. 1. Novel *BCKDHA* and *BCKDHB* mutations detected in this study among Indian MSUD patients. A: c.1249delC (family 2); (B) c.1312T>C (p.Y438H from family 3); (C) c.1561T>A (family 4) (all in *BCKDHA*); (D) c.548G>A (p.R183Q from family 8); (E) c.964A>G (p.T322A from family 8); (F) c.401T>A (p.I134N from family 9) and (G) c.1065delT (family 9) (all in *BCKDHB*). For each, electropherogram showing the mutation is on the left and the one showing the normal sequence is on the right. For G, electropherogram on the left represents the patient's heterozygous mutant sequence while the one in the middle and the right represent mutant and normal sequence of cloned PCR products, respectively. The mutated residue is indicated by an arrow; the deleted residues in A and G are indicated in the normal sequence. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]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gi|148727347|ref|NP_001092034.1 LRKQQESLARHLQTYGEHYPLDHFDK
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gi|332242782|ref|XP_003270562.1 LRKQQESLARHLQTYGEHYPLEHFDK
gi|296233895|ref|XP_002762220.1 LRKQQESLARHLQTYGEHYPLEHFDK
gi|73947481|ref|XP_866392.1 LRKQQEALARHLQTYGEHYPLDHFEK
gi|301776619|ref|XP_002923727.1 LRKQQEALARHLQTYGEHYPLDHFEK
gi|338710481|ref|XP_001500344.2 LRKQQESLARHLQTYGEHYPLDHFEK
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116
155
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Fig. 2.

SEQUENCE AND STRUCTURE ANALYSIS

Sequence analysis of each mutant amino acid residue was performed essentially as described earlier [Bashyam et al., 2012]; details are given in Supplementary Methods S1.

RESULTS

Mutations detected in the nine affected families are given in Table IIA. Four families harbored mutations in *BCKDHA* while the other five harbored mutations in *BCKDHB*. Three of the four *BCKDHA* families harbored novel mutations (Fig. 1 and Table IIA); homozygous mutations in three (family 1–3; Table IIA) and heterozygous in one (family 4; Table IIA). We could not detect the second mutation in proband of family 4. Since there is no earlier evidence for autosomal dominant mode of inheritance in MSUD and the father of the proband harboring the c.1561T>A heterozygous mutation was clinically normal, it is likely that the second mutation was inherited from the mother and is expected to be located in intronic regions. Among the five *BCKDHB* families, three harbored homozygous mutation (families 5, 6, and 7; Table IIA). Probands from families 8 and 9, exhibited compound heterozygosity and harbored novel mutations (Fig. 1 and Table IIA). Overall, we identified seven novel mutations out of the total 11 mutations identified.

Multiple sequence alignments of E1α and E1β with their respective homologues (Fig. 2A,B) as well as analysis of the position-specific profile Gribskov's scores (Table IIB) confirmed high evolutionary conservation of all affected residues; substitutions at these positions are therefore expected to be unfavorable. E1α and E1β regions located in the interface of the multimeric enzyme complex are shown in Tables IIIA and IIIB, respectively. More importantly, the recently developed web-server Hansa (hansa.cdfd.org.in:8080) [Acharya and Nagarajaram, 2012; Bashyam et al., 2012] predicted all missense mutations as "Disease" (Table IIB).

The E1α R346C mutation is expected to disrupt the hydrogen bonding network destabilizing the structure of the phosphorylation loop [Li et al., 2004]. The p.Y438H mutation appears to preclude H-bond interaction of Y438 with side chains of E1α H430 and E1β D378 (Fig. 3A) which in turn is expected to destabilize the structure

TABLE III. Amino Acid Residues of α/α′ (A) and β/β′ (B) Buried Due to Subunit Association $\alpha^2\beta^2$

Amino acid residue position	Residue type	Percent solvent accessible area of residues: In the complex $\alpha^2\beta^2$	Percent solvent accessible area of residues: In the isolated α/α′ subunit
A			
52	PRO*	16.01	19.22
53	GLN*	43.85	51.49
54	PHE*	3.66	44.91
55	PRO*	23.28	43.86
56	GLY*	1.42	23.5
57	ALA*	5	23.16
58	SER*	15	33.51
59	ALA*	4.68	22.31
60	GLU*	32.24	43.52
61	PHE*	19.31	47.52
62	ILE*	13.13	36.62
63	ASP*	12.58	36.49
64	LYS*	29.16	38.23
65	LEU*	13.08	48.19
66	GLU*	24.47	28.84
67	PHE*	6.99	56.81
68	ILE*	19.63	52.81
69	GLN*	38.38	45.35
70	PRO*	6.78	30.93
72	VAL*	15.05	19.12
73	ILE*	48.57	56.05
74	SER*	16.72	22.37
75	GLY*	3.02	7.18
76	ILE*	8.09	17.84
77	PRO*	13.11	35.94
78	ILE*	0.03	29.49
79	TYR*	0.03	15.21
80	ARG*	17.23	36.91
89	ILE*	17.76	23.98
90	ASN*	8.23	21.07
92	SER*	29.35	29.38
93	GLU*	8.84	18.19
129	SER***	11.16	26.51
130	PHE***	11.53	14.95
137	GLU	1.54	1.6
153	LEU**	2.82	8.91
155	PHE **	0.51	4.36
156	GLY	0.7	0.98
157	GLN	2.02	2.08
158	TYR***	4.45	14.04
159	ARG	13.23	20.41
177	GLN***	2.92	3.23
178	CYS***	0.37	1.45
179	TYR***	4	6.45
180	GLY***	0	4.27
185	LEU**	34.03	48.59

(*Continued*)

Fig. 2. (*Overleaf*) A: Multiple sequence alignment (MSA) of human *BCKDHA* with homologues from other species. The MSA was performed as described in the Materials and Methods Section. The position of each mutated residue is shown by an arrow. amino acid positions corresponding to the human sequence are indicated above each alignment. The homologues are as follows: gi|258645170|ref|NP_000700.1|, *Homo sapiens*; gi|77736548|ref|NP_036914.1|, *Rattus norvegicus*; gi|183396774|ref|NP_031559.3|, *Mus musculus*; gi|148727347|ref|NP_001092034, *Pan troglodytes*; gi|62510814|sp|Q8HXY4.1|ODBA_M, *Macaca fascicularis*; gi|297277135|ref|XP_001101959, *Macaca mulatta*; gi|332242782|ref|XP_003270562, *Nomascus leucogenys*; *gi*|296233895|ref|XP_002762220, *Callithrix jacchus*; gi|73947481|ref|XP_866392.1|, *Canis familiaris*; gi|301776619|ref|XP_002923727, *Ailuropoda melanoleuca*; *gi*|338710481|ref|XP_001500344, *Equus caballus*; *gi*|187607469|ref|NP_001119816, *Ovis aries*; gi|27806229|ref|NP_776931.1|, *Bos taurus*; gi|178056466|ref|NP_001116555, *Sus scrofa*; *gi*|291412159|ref|XP_002722340, *Oryctolagus cuniculus*; gi|126329384|ref|XP_001372218, *Monodelphis domestica*; gi|327276395|ref|XP_003222955, *Anolis carolinensis*; gi|66773104|ref|NP_001019590.1, *Danio rerio*; gi|195395472|ref|XP_002056360, *Drosophila virilis*. B: Multiple sequence alignment (MSA) of human *BCKDHB* with homologues from other species. The MSA was performed as described in the Materials and Methods Section. The position of each mutated residue is shown by an arrow. amino acid positions corresponding to the human sequence are indicated above each alignment. The homologues are as follows: gi|4557353|ref|NP_000047.1|, *Homo sapiens*; gi|162416262|sp|Q6P3A8.2|, *Mus musculus*; gi|334324067|ref|XP_001375236, *Monodelphis domestica*; gi|348578059|ref|XP_003474801, *Caria porcellus*; gi|301761846|ref|XP_002916344, *Ailuropoda melanoleuca*; gi|348532057|ref|XP_003453523, *Oreochromis niloticus*; gi|198285569|gb|ACH85323.1|, *Salmo salar*; gi|115502434|sp|P21839.2|, *Bos tarus*; gi|332218346|ref|XP_003258317, *Nomascus leucogenys*; gi|178056478|ref|NP_001116691, *Sus scrofa*; gi|73973855|ref|XP_532213.2|, *Canis lupus familiaris*; gi|291396526|ref|XP_002714592, *Oryctolagus cuniculus*; gi|158749538|ref|NP_062140.1|, *Rattus norvegicus*; gi|344264129|ref|XP_003404146, *Loxodonta africana*.

TABLE III. (*Continued*)

Amino acid residue position	Residue type	Percent solvent accessible area of residues: In the complex $\alpha^2\beta^2$	Percent solvent accessible area of residues: In the isolated α/α' subunit
186	GLY**	4.5	13.41
187	LYS**/***	15.06	21.23
188	GLY***	0.05	8.26
189	ARG**/***	0.04	30.16
190	GLN**/***	1.67	21.28
191	MET***	1.98	29.46
192	PRO***	0	2.76
196	GLY**	3.03	6.61
197	CYS**	2.91	5.08
198	LYS**	36.96	58.16
201	HIS**	15.2	15.76
202	PHE**	0.38	1.17
203	VAL**	1.12	16.99
204	THR**	2.81	23.08
205	ILE**	0.64	0.69
206	SER**/***	0.91	14.27
207	SER***	0.48	14.58
208	PRO**/***	0.37	26.43
209	LEU***	3.09	28.68
211	THR**	0.41	12.68
212	GLN**	3.28	4.45
214	PRO**	0.06	12.94
215	GLN**	0.2	30.25
217	VAL**	0.02	1.2
218	GLY**	0	13.16
219	ALA**	0.09	7.89
221	TYR**	2.82	24.43
222	ALA**	3.14	19.08
223	ALA**	3.36	3.78
224	LYS**	16.19	16.27
225	ARG**	21.72	55.67
230	ARG**	18.36	22.5
237	GLY	0.34	3.35
238	GLU	0.05	0.67
239	GLY***	0	4.11
240	ALA***	0.46	1.72
241	ALA*	0.15	1.79
242	SER*/***	0.18	17.53
243	GLU*/***	1.87	21.68
244	GLY*/**/***	0.1	17.84
245	ASP**/***	3.06	13.59
246	ALA*	0.33	0.63
247	HIS*/**	0.95	34.9
248	ALA*/**	0.08	10.27
251	ASN*/**	0.37	17.97
252	PHE*/**	0.15	34.69
254	ALA*	1.7	6.88
255	THR*/**	2.12	28.15
256	LEU**	5.94	26.75
257	GLU*	20.57	27.48
258	CYS**	0	0.01
265	ARG	1.62	7.03
266	ASN*	0.12	0.81
267	ASN*	2.12	7.7
268	GLY*	0.15	6.7
269	TYR*	15.94	21.58
270	ALA***	0.77	9.86
271	ILE***	21.8	55.67
272	SER***	8.18	26.47
273	THR***	1.19	10.92
274	PRO***	12.85	12.96
275	THR*	2.17	6.89
276	SER*	20.61	20.87
277	GLU*/***	11.34	35
278	GLN*/***	0.42	10.34
279	TYR*/***	3.12	12.69
280	ARG*	26.13	59.71
281	GLY*	0.91	11.64
282	ASP*	2.6	4.94
283	GLY*	0.18	2.46
286	ALA*	7.96	12.46

(*Continued*)

TABLE III. (*Continued*)

Amino acid residue position	Residue type	Percent solvent accessible area of residues: In the complex $\alpha^2\beta^2$	Percent solvent accessible area of residues: In the isolated α/α' subunit
287	ARG*	3.84	21.58
289	PRO*	8.03	25.03
290	GLY*	2.17	22.1
291	TYR*	2.76	14.01
292	GLY*	4.4	16.99
293	ILE*	0.05	0.79
294	MET*	4.27	26.47
295	SER*	0	9.55
296	ILE*	1.71	21.91
297	ARG*	0.05	4.34
299	ASP*	0.25	4.92
301	ASN*	0.32	1.71
308	ASN*	5.37	8.19
309	ALA*	0	2.26
312	GLU*	12.36	22.59
313	ALA*	0	1.44
316	ARG*	14.49	17.78
324	PHE*	0	5.19
325	LEU*	0.09	0.27
326	ILE*	0.01	0.33
329	MET*	0.03	17.21
330	THR*	0.05	2.86
331	TYR*	19.54	38.91
332	ARG	51.01	51.11
358	ASP*	22.64	22.79
363	ARG*	1.91	1.97
402	LYS***	11.24	16.48
403	PRO***	1.5	4.81
404	ASN***	11.26	11.65
405	PRO***	3.01	10.86
407	LEU***	17.59	18.84
408	LEU**/***	0	34.38
409	PHE***	0.17	5.44
410	SER**	9.3	9.89
411	ASP**	4.32	21.72
412	VAL**/***	0.84	28.45
413	TYR**/***	3.51	37.63
414	GLN**	26.69	52.76
415	GLU**	25.48	34.72
417	PRO**	6.52	14.96
419	GLN**/***	12.35	37.4
420	LEU***	2.2	10.12
422	LYS**	29.84	37.08
423	GLN***	6.29	29.65
426	SER***	11.45	14.04
427	LEU***	1.36	14.62
429	ARG***	43.8	47.23
430	HIS***	4.57	21.33
431	LEU***	7	8.65
433	THR***	27.17	27.8
434	TYR***	5.66	30.77
437	HIS***	23.07	38.14
438#	TYR***	4.11	25.18
439	PRO***	12.19	22.27
440	LEU***	12.31	13.69
443	PHE***	4.97	19.92

Amino acid residue position	Residue type	Percent solvent accessible areas: In the complex $\alpha^2\beta^2$	Percent solvent accessible areas: In the isolated β/β' subunit
B			
89	PRO	32.95	36.2
90	THR**	18.23	20.38
92	VAL**	1.16	7.37

(*Continued*)

TABLE III. (*Continued*)

Amino acid residue position	Residue type	Percent solvent accessible areas: In the complex $\alpha^2\beta^2$	Percent solvent accessible areas: In the isolated β/β' subunit
96	GLU***	3.83	4.16
97	ASP***	1.13	16.2
99	ALA***	5.66	6.37
100	PHE***	37.1	46.41
103	VAL***	15.04	20.75
111	ARG***	20.19	24.21
116	LYS**	44.19	44.2
117	ASP**	17.83	30.68
118	ARG**	7.57	11.51
120	PHE**	3.08	13.69
121	ASN**/***	1.96	22.26
122	THR**/***	0.36	7.34
123	PRO**/***	0.02	36.62
124	LEU***	5.65	46.51
125	CYS**/***	0.15	17.11
126	GLU***	1.5	4.96
127	GLN*/**/***	2.84	30.74
128	GLY**	0	10.14
129	ILE**	0.18	0.85
131	GLY**	0	6.44
132	PHE**	0.03	30.62
134#	ILE**	0	2.1
135	GLY**	0	11.48
136	ILE**	0.64	10.46
138	VAL**	5.3	14.8
139	THR**	14.42	33.96
141	ALA**	4.89	6.87
148	GLN***	3.98	7.39
151	ASP*/***	0	1.73
152	TYR*/***	9.5	23.35
154	PHE*	0	21.13
155	PRO*/***	7.88	22.2
157	PHE*	0	20.47
158	ASP*/**	2.48	24.31
159	GLN**	0.5	15.82
161	VAL*	0.32	10.1
162	ASN*/**	0	21.79
163	GLU*/**	0.26	13.57
165	ALA*/**	0.53	2.19
166	LYS*	3.12	23.91
167	TYR**	0.54	10.35
168	ARG**	5.8	26.48
169	TYR*/**	0.02	58.35
170	ARG*/**	1.73	36.54
171	SER**	0.57	8.65
172	GLY**	1.53	19.33
173	ASP**	16.4	23.33
174	LEU*/**	10.61	45.74
175	PHE**	4.98	35.06
178	GLY	0.5	0.53
179	SER	6.74	6.82
180	LEU	0.07	0.14
181	THR	0.15	0.19
191	HIS***	25.91	38.01
192	GLY***	0.01	2.37
193	ALA***	0.5	7.2
194	LEU***	0.39	21.45
195	TYR*/***	0.81	37.68
196	HIS***	10.43	22.07
198	GLN*	6.56	13.12
199	SER*	2.92	4.04
200	PRO*	0.23	0.35
202	ALA*	2.49	7.12
203	PHE*	3.05	37.26
205	ALA*	0.65	5.84
206	HIS*	1.79	49.82
207	CYS*	0.31	1.44
208	PRO*/**	5.25	36.43
209	GLY**	0.32	12.94
210	ILE**	0.52	1.45
211	LYS**	1.92	9.47

(*Continued*)

TABLE III. (*Continued*)

Amino acid residue position	Residue type	Percent solvent accessible areas: In the complex $\alpha^2\beta^2$	Percent solvent accessible areas: In the isolated β/β' subunit
228	CYS	0	0.07
229	ILE	2.56	2.64
231	ASP	7.53	7.79
232	LYS	36.33	47.22
233	ASN**	0.46	12.78
308	ILE*/**	2.65	7.39
309	PRO**	7.33	22.97
310	TRP*/**	2.64	9.25
312	VAL**	2.47	15.11
313	ASP**	29.41	31.02
331	ALA*	0.67	1.93
332	PRO*	7.64	17.46
333	LEU*/***	13.52	19.1
334	THR*	6.59	31.49
335	GLY*	5.41	17.32
336	GLY*	0.28	0.94
337	PHE*	1.14	2.71
339#	SER*	0.54	11.92
340	GLU*	0.21	21.43
342	SER*	0.04	5.4
343	SER*	0.16	15.34
344	THR*/**	0.8	5.67
346	GLN*	0.26	20.96
347	GLU*	13.62	32.79
348	GLU**	23.42	29.78
350	PHE*	8.74	40.53
351	LEU*	45.73	53.44
353	LEU*	4.86	12.12
354	GLU*	28.27	29.84
355	ALA*	0	1.81
356	PRO*	4.8	22.1
357	ILE*	0.08	7.01
358	SER*	6.58	7.24
359	ARG*	6.13	25.71
363	TYR***	5.29	33.69
364	ASP*/***	5.92	23.56
365	THR*/***	0.58	22.31
366	PRO*/***	1.97	30.48
367	PHE*/***	4.94	5.12
368	PRO*/***	0	7.87
369	HIS***	3.36	31.27
370	ILE***	9.9	39.64
371	PHE*/***	0	38.54
373	PRO***	16.35	27.37
374	PHE***	0	46.57
375	TYR***	0.47	2.36
378	ASP***	6.29	10.17
380	TRP***	12.3	43.92
381	LYS***	6.73	20.06
383	TYR***	3.72	7.08
384	ASP***	4.63	15.31
387	ARG***	24.52	25.9
388	LYS***	32.25	35.62
392	TYR*	35.11	50.97

#, mutated residues; *, residues present at homodimers interface of α–α' and β–β' subunits; **, residues present at interface of α–β; ***, residues present at α–β' and β–α' subunits; /, residues present at more than one interface.

of E1α as well as E1α–E1β interaction. The E1β I134 is located in a helix (residues 126–138) where its main chain C=O and N–H groups are involved in helical hydrogen bonds (backbone–backbone hydrogen bonds) with other residues in the helix viz., V130, A137, and V138 (Fig. 3B). N134 is expected to make an additional H-bond with V130 (side chain to main chain) (Fig. 3B) thus possibly weakening the helical H-bond which in turn can destabilize the helix. Furthermore, this helix is at the interface region of E1α–E1β complex and hence the mutation is expected to impede E1α–E1β

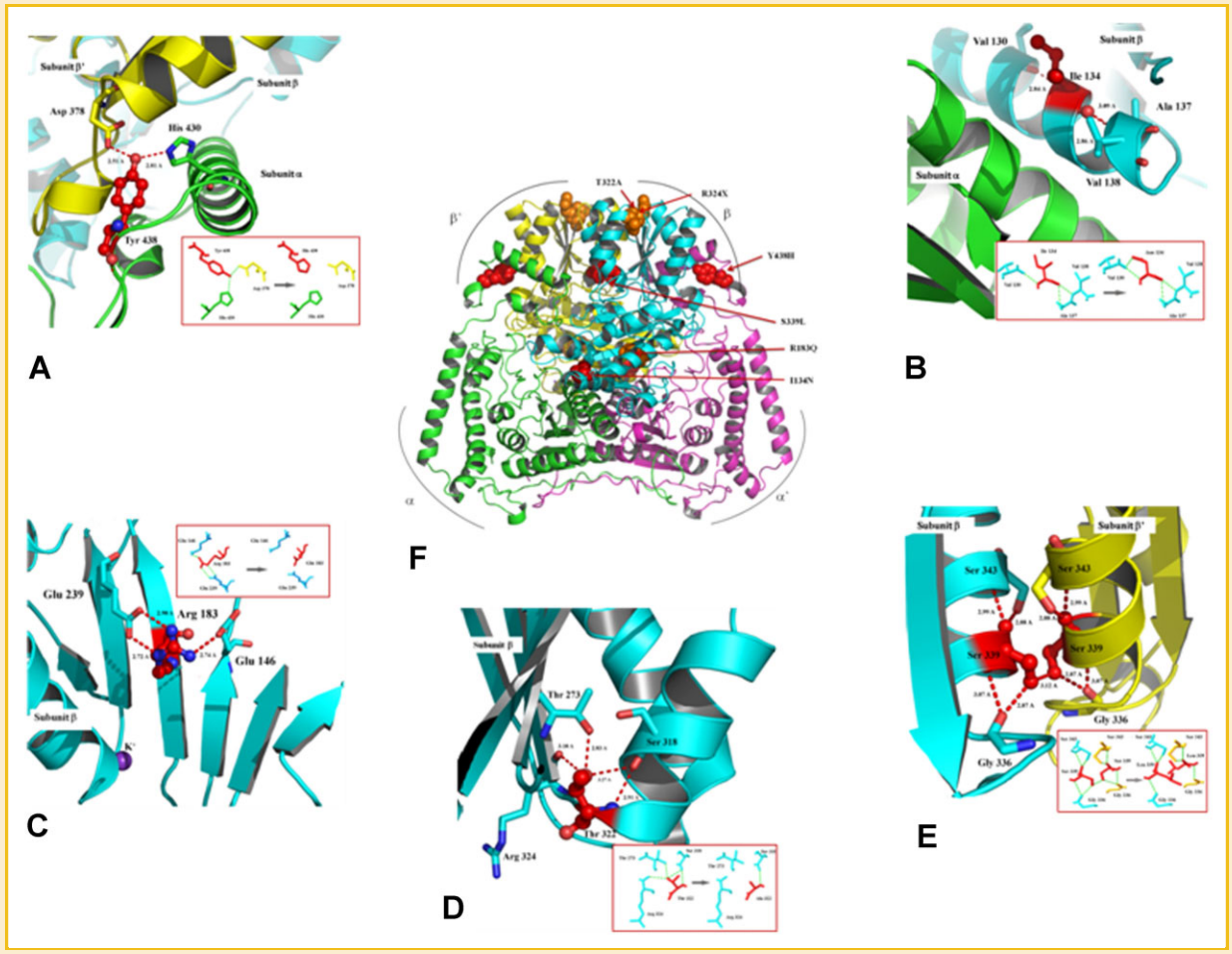

Fig. 3. Structure analysis of missense mutations identified in this study. Panel A depicts the effect of *BCKDHA* p.Y438H mutation; subunit α is shown in green, β in cyan, β′ in yellow and the mutated residue in red ball and stick model. Hydrogen bonds are denoted by red dotted lines. Panels B to E depict effects of *BCKDHB* mutations I134N (B), R183Q (C), T322A (D) and S339L (E); color schema is same as in panel A. Panel F shows a ribbon-plot representation of the E1 heterotetramer showing the location of mutated amino acid residues; E1α′ is depicted in magenta. Missense mutations identified in E1α and E1β are represented by spheres. Mutations occurring at interface region are shown in red and other mutations are in orange. The ribbon-plot was generated using PyMOL [DeLano, 2002] (DeLano Scientific, San Carlos, CA) using the Protein Data Bank entry 1X7Y. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

assembly. The R183 residue, located in a beta sheet that includes a K+ ion-binding pocket (Fig. 3C) appears to form salt bridges with Glu239 and Glu146 (Fig. 3C). Glutamine at this position may lead to disruption of the salt-bridges (Fig. 3C, inset) as well as K+ binding. The T322 residue is located at the C-terminal end of a helix (residues 312–322) and is expected to make side chain H-bonds with R334, Y273, and S318 in addition to a main chain H-bond with S318 (Fig. 3D). All three side chain H-bonds are lost in the mutant A322 protein (Fig. 3D, inset) which may result in destabilization of the E1β structure. S339 is involved in side chain-side chain H-bonding with S339 of another E1β subunit that may be important for dimerization (Fig. 3E). Substitution of a bulkier hydrophobic residue like leucine at this position precludes side chain H-bonding (Fig. 3E, inset) and hampers tight packing of the two beta subunits.

Family 2 harbored the *BCKDHA* c.1249delC mutation (located in the last (9th) exon); the mutation results in a change in amino acid sequence starting from 417th residue and causes addition of 38 extra amino acids (Fig. 4A). The mutation is expected to perturb E1α–E1β interaction. Family 4 harbored the *BCKDHA* c.1561T>A heterozygous mutation located in the 3′-UTR (Fig. 4B). The mutation may perturb mRNA stability, transport or translation efficiency. The mutation does not appear to perturb any known miRNA target sequence (data not shown). This is the first 3′-UTR mutation detected in either *BCKDHA* or *BCKDHB* though it was previously reported in *DBT* [Brodtkorb et al., 2010]. Family 5 harbored the *BCKDHB* c.853C>T (p.R285X) nonsense mutation that generates a premature termination codon (PTC) 99 nucleotides upstream of the exon 8–exon 9 junction (Fig. 4C). The PTC did not alter normal splicing of the exon (data not shown). The mutation however resulted in a significant reduction in *BCKDHB* transcript level when compared with wild type *BCKDHB* transcript (Fig. 4D) suggesting that the mutant transcript was subjected to nonsense mediated decay (NMD) induced degradation [Nagy and Maquat, 1998; Bashyam, 2009]. In addition, the residual truncated protein (synthesized on the minor intact mRNA fraction) would be devoid of C-terminal 107 amino acid residues which include the E1β interface segment (aa residues

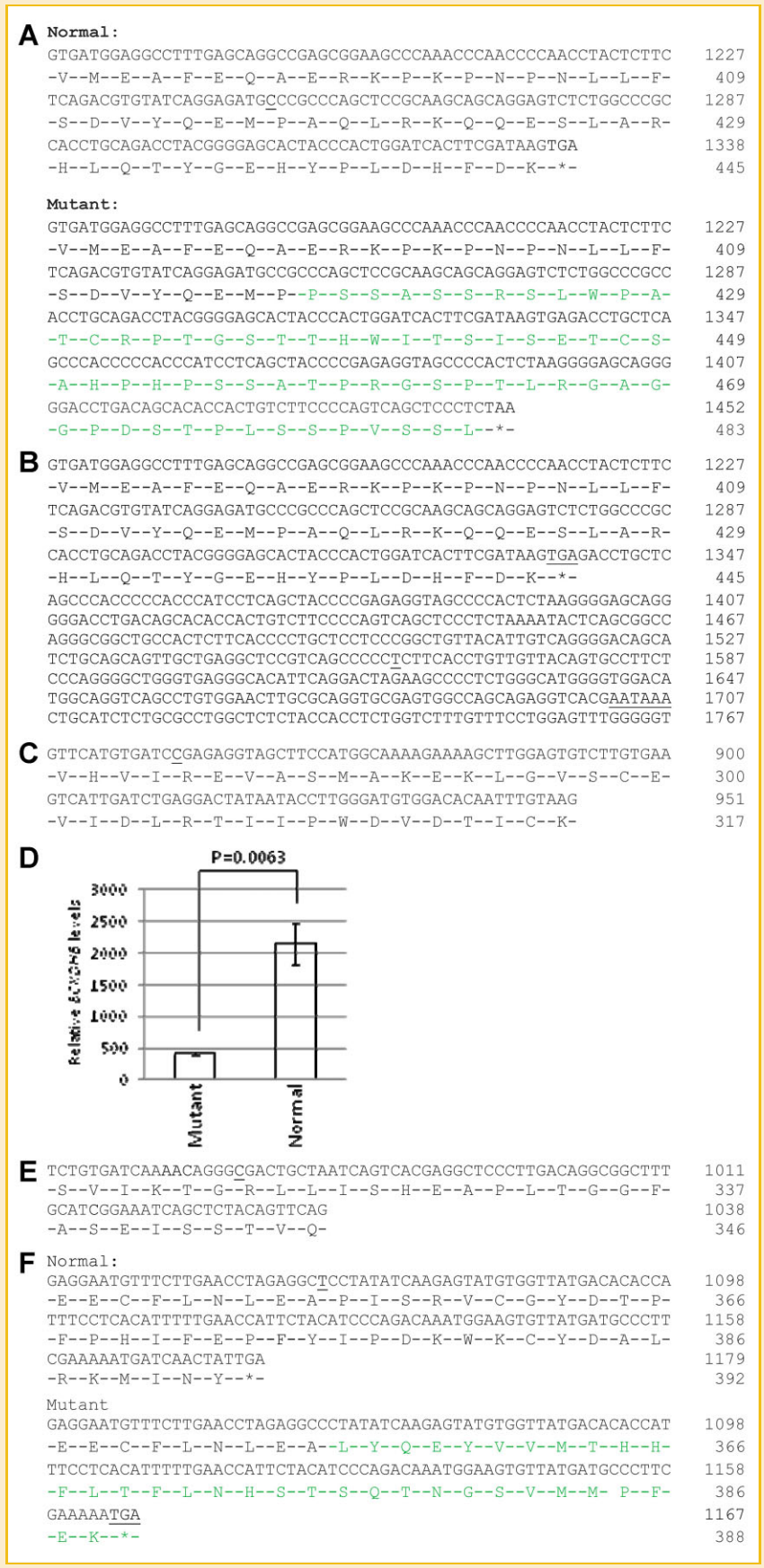
A
Normal:
GTGATGGAGGCCTTTGAGCAGGCCGAGCGGAAGCCCAAACCCAACCCCAACCTACTCTTC 1227
-V--M--E--A--F--E--Q--A--E--R--K--P--K--P--N--P--N--L--L--F- 409
TCAGACGTGTATCAGGAGATGCCGCCCAGCTCCGCAAGCAGCAGGAGTCTCTGGCCCGC 1287
-S--D--V--Y--Q--E--M--P--A--Q--L--R--K--Q--Q--E--S--L--A--R- 429
CACCTGCAGACCTACGGGGAGCACTACCCACTGGATCACTTCGATAAGTGA 1338
-H--L--Q--T--Y--G--E--H--Y--P--L--D--H--F--D--K--*- 445
Mutant:
GTGATGGAGGCCTTTGAGCAGGCCGAGCGGAAGCCCAAACCCAACCCCAACCTACTCTTC 1227
-V--M--E--A--F--E--Q--A--E--R--K--P--K--P--N--P--N--L--L--F- 409
TCAGACGTGTATCAGGAGATGCCGCCCAGCTCCGCAAGCAGCAGGAGTCTCTGGCCCGCC 1287
-S--D--V--Y--Q--E--M--P--P--S--S--A--S--S--R--S--L--W--P--A- 429
ACCTGCAGACCTACGGGGAGCACTACCCACTGGATCACTTCGATAAGTGAGACCTGCTCA 1347
-T--C--R--P--T--G--S--T--T--H--W--I--T--S--I--S--E--T--C--S- 449
GCCCACCCCCACCCATCCTCAGCTACCCCGAGAGGTAGCCCCACTCTAAGGGGAGCAGGG 1407
-A--H--P--H--P--S--S--A--T--P--R--G--S--P--T--L--R--G--A--G- 469
GGACCTGACAGCACACCACTGTCTTCCCCAGTCAGCTCCCTCTAA 1452
-G--P--D--S--T--P--L--S--S--P--V--S--S--L--*- 483
B
GTGATGGAGGCCTTTGAGCAGGCCGAGCGGAAGCCCAAACCCAACCCCAACCTACTCTTC 1227
-V--M--E--A--F--E--Q--A--E--R--K--P--K--P--N--P--N--L--L--F- 409
TCAGACGTGTATCAGGAGATGCCCGCCCAGCTCCGCAAGCAGCAGGAGTCTCTGGCCCGC 1287
-S--D--V--Y--Q--E--M--P--A--Q--L--R--K--Q--Q--E--S--L--A--R- 429
CACCTGCAGACCTACGGGGAGCACTACCCACTGGATCACTTCGATAAGTGAGACCTGCTC 1347
-H--L--Q--T--Y--G--E--H--Y--P--L--D--H--F--D--K--*- 445
AGCCCACCCCCACCCATCCTCAGCTACCCCGAGAGGTAGCCCCACTCTAAGGGGAGCAGG 1407
GGGACCTGACAGCACACCACTGTCTTCCCCAGTCAGCTCCCTCTAAAATACTCAGCGGCC 1467
AGGGCGGCTGCCACTCTTCACCCCTGCTCCTCCCGGCTGTTACATTGTCAGGGGACAGCA 1527
TCTGCAGCAGTTGCTGAGGCTCCGTCAGCCCCCTCTTCACCTGTTGTTACAGTGCCTTCT 1587
CCCAGGGGCTGGGTGAGGGCACATTCAGGACTAGAAGCCCCTCTGGGCATGGGGTGGACA 1647
TGGCAGGTCAGCCTGTGGAACTTGCGCAGGTGCGAGTGGCCAGCAGAGGTCACGAATAAA 1707
CTGCATCTCTGCGCCTGGCTCTCTACCACCTCTGGTCTTTGTTTCCTGGAGTTTGGGGGT 1767
C
GTTCATGTGATCCGAGAGGTAGCTTCCATGGCAAAAGAAAAGCTTGGAGTGTCTTGTGAA 900
-V--H--V--I--R--E--V--A--S--M--A--K--E--K--L--G--V--S--C--E- 300
GTCATTGATCTGAGGACTATAATACCTTGGGATGTGGACACAATTTGTAAG 951
-V--I--D--L--R--T--I--I--P--W--D--V--D--T--I--C--K- 317
D
P=0.0063
3000
2500
2000
1500
1000
500
0
Mutant
Normal
E
TCTGTGATCAAAACAGGGCGACTGCTAATCAGTCACGAGGCTCCCTTGACAGGCGGCTTT 1011
-S--V--I--K--T--G--R--L--L--I--S--H--E--A--P--L--T--G--G--F- 337
GCATCGGAAATCAGCTCTACAGTTCAG 1038
-A--S--E--I--S--S--T--V--Q- 346
F
Normal:
GAGGAATGTTTCTTGAACCTAGAGGCTCCTATATCAAGAGTATGTGGTTATGACACACCA 1098
-E--E--C--F--L--N--L--E--A--P--I--S--R--V--C--G--Y--D--T--P- 366
TTTCCTCACATTTTTGAACCATTCTACATCCCAGACAAATGGAAGTGTTATGATGCCCTT 1158
-F--P--H--I--F--E--P--F--Y--I--P--D--K--W--K--C--Y--D--A--L- 386
CGAAAAATGATCAACTATTGA 1179
-R--K--M--I--N--Y--*- 392
Mutant
GAGGAATGTTTCTTGAACCTAGAGGCCCTATATCAAGAGTATGTGGTTATGACACACCAT 1098
-E--E--C--F--L--N--L--E--A--L--Y--Q--E--Y--V--V--M--T--H--H- 366
TTCCTCACATTTTTGAACCATTCTACATCCCAGACAAATGGAAGTGTTATGATGCCCTTC 1158
-F--L--T--F--L--N--H--S--T--S--Q--T--N--G--S--V--M--M- P--F- 386
GAAAAATGA 1167
-E--K--*- 388

Fig. 4.

331–392) thereby perturbing proper E1α–E1β interaction. Family 6 harbored the *BCKDHB* c.970C>T (p.R324X) nonsense mutation generating a PTC located 69 bp upstream of the exon 9–exon 10 junction (Fig. 4E) which is expected to trigger NMD. Similar to the p.R285X mutation, the truncated protein synthesized on residual mutant transcript will be devoid of the C-terminal 68 amino acids and is expected to perturb E1α–E1β interaction. Family 9 harbored the *BCKDHB* c.1065delT mutation located in the last exon (exon 10) resulting in a change in amino acid sequence from position 355 (Fig. 4F) thereby perturbing the residues important for interaction with E1α subunit. The altered reading frame results in a PTC at amino acid position 388 (Fig. 4F).

DISCUSSION

This is the first molecular genetic analysis of MSUD from the Indian population. The fact that we identified disease causing mutations in all patients reveals that *BCKDHA* and *BCKDHB* could be the major genes causing MSUD in the Indian population. Our results have revealed an approximately equal frequency in the two genes as reported in previous studies [Nellis and Danner, 2001; Flaschker et al., 2007]. In addition, 64% (7/11) of mutations were novel indicating a unique mutation pattern in the Indian population as reported for other genetic disorders [Bashyam et al., 2010, 2012]. The *BCKDHA* R346 amino acid residue has been shown to be affected previously in MSUD viz. p.R346H [Rodriguez-Pombo et al., 2006] and p.R346C [Park et al., 2011]. The *BCKDHA* Y438 residue is perhaps the most frequently affected residue in MSUD patients [Brunetti-Pierri et al., 2011; Nellis and Danner, 2001; Henneke et al., 2003]; though it was also incorrectly reported as Y394 [Zhang et al., 1989] and Y393 [Fisher et al., 1991]. Similarly, the *BCKDHB* R183P mutation was identified in previous studies [Edelmann et al., 2001; Gorzelany et al., 2009] though incorrectly reported as R133P in one [Wynn et al., 2001]. The *BCKDHB* p.R285X mutation was previously identified from Turkey [Henneke et al., 2003]. The *BCKDHB* R324X mutation was identified earlier [Edelmann et al., 2001; Nellis et al., 2003] and incorrectly reported as R274X [McConnell et al., 1997]. The *BCKDHB* S339L mutation was also reported previously [Gorzelany et al., 2009] though incorrectly reported as S289L [Wynn et al., 2001].

Absence of each novel mutation in at least 50 healthy individuals from the local population was confirmed. Mutations occurring in exons 6 and 7 of *BCKDHA* account for about half of all mutations listed in HGMD while all *BCKDHA* mutations detected in this study localized to the 8th and 9th exons and to the 3′-UTR. Similarly, exons 4, 5, and 6 of *BCKDHB* harbor more than 60% of mutations listed in the HGMD, while we identified only one mutation in exon 4, one in exon 5 and rest of the five mutations were detected in exons 8, 9, and 10. Therefore, a majority of mutations identified in this study localize to the C-terminal end of E1α and E1β resulting probably in disruption of the $\alpha^2\beta^2$ complex.

Of the 11 mutations identified, four appeared to result in a truncated protein; one in *BCKDHA* and three in *BCKDHB* (Table IIA). Among these, two were nonsense mutations (both in *BCKDHB*) while the other two were single base deletion mutations (one each in the two genes), which generated PTC due to a change in the reading frame. The *BCKDHB* c.853C>T (p.R285X) mutation appeared to induce degradation of the transcript due to NMD (Fig. 4C,D) and the c.970C>T (p.R324X) mutation (Fig. 4F) is also expected to trigger NMD. There is only one previous report of NMD in MSUD, validated in the BCKDHA [Fernandez-Guerra et al., 2010]. The BCKDHA c.1249delC mutation located in the last exon results in addition of 38 amino acids to the C-terminal end of the protein (Fig. 4A). A complex *BCKDHA* mutation located between nucleotide positions 1233 and 1243 was reported earlier to result in addition of 37 extra amino acids at the C-terminus [Rodriguez-Pombo et al., 2006].

In the current study we evaluated nine MSUD patients from India and identified seven novel mutations. The study revealed a high frequency of mutations causing altered protein truncation that perturb the C-termini of E1α and E1β possibly disrupting E1 assembly. The study is the first step towards identification of mutation spectrum in the Indian population and has important implications for patient management and genetic counseling.

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Fig. 4. Depiction of effect of nonsense, single base deletion and 3′-UTR mutations. Panel A depicts effect of the *BCKDHA* c.1249delC mutation; both the nucleotide and amino acid sequences are shown. The deleted "C" residue is underlined in the normal sequence. The altered amino acid residues generated due to the deletion are shown in green in the mutant sequence. Panel B depicts effect of the *BCKDHA* c.1561T>A 3′-UTR mutation. The position of the mutated "T" residue (underlined) with respect to the termination codon (TGA, underlined) and the poly A sequence (AATAAA, underlined) is indicated. Panel C depicts effect of the *BCKDHB* c.853C>T mutation; the complete sequence of exon 8 is shown. The mutated "C" residue is underlined; the mutation results in generation of a PTC (TGA) located 99 nucleotides upstream of exon 8–exon 9 junction. Panel D shows the result of quantitative RT-PCR based evaluation of *BCKDHB* transcript level relative to *GAPDH* in RNA isolated from fibroblasts derived from skin biopsy obtained from a normal individual and the proband from family 5 harboring the c.853C>T mutation. The *P* value corresponds to an unpaired *t* test. Panel E depicts the effect of the *BCKDHB* c.970C>T mutation; the mutated "C" residue is underlined. The mutation results in generation of a PTC (TGA) in the 9th exon located 69 nucleotides upstream of exon 9–exon 10 junction. Panel F depicts the effect of the *BCKDHB* c.1065delT mutation; the deleted "T" nucleotide is underlined in the normal sequence. In the mutant sequence, the altered amino acid residues generated due to the deletion are shown in green. The PTC generated eight nucleotides upstream of the authentic termination codon is underlined in the mutant sequence.

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