

# In silico analysis of novel mutations in maple syrup urine disease patients from Iran

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**Abstract** Maple Syrup Urine Disease (MSUD) is a rare autosomal recessive disorder of branched-chain amino acid (BCAA) metabolism. The disease is mainly caused by mutations either in the *BCKDHA*, *BCKDHB*, *DBT* or *DLD* genes encoding components of the E1 $\alpha$ , E1 $\beta$ , E2 and E3 subunits of branched-chain  $\alpha$ -keto acid dehydrogenase complex (BCKDC), respectively. BCKDC is a mitochondrial enzyme which is responsible for the normal breakdown of BCAA. The rate of consanguineous marriage in Iran is 38.6 %, so the prevalence of autosomal recessive disorders is higher in comparison to other countries. Consanguinity increases the chance

of the presence of pathogenic mutations in a homoallelic state. This phenomenon has made homozygosity mapping a powerful tool for finding the probable causative gene in heterogeneous disorders like IEM (Inborn Errors of Metabolism). In this study, two sets of multiplex polymorphic STR (Short Tandem Repeat) markers linked to the above-mentioned genes were selected to identify the probable pathogenic gene in the studied families. The families who showed a homozygous haplotype for the STR markers of the *BCKDHB* gene were subsequently sequenced. Four novel mutations including c.633 + 1G > A, c.988G > A, c.833\_834insCAC, and a homozygous deletion of whole exon 3 c. (274 + 1\_275–1)\_(343 + 1\_344–1), as well as one recently reported (c. 508G > T) mutation have been identified. Interestingly, three families shared a common haplotype structure along with the c. 508G > T mutation. Also, four other families revealed another similar haplotype with c.988G > A mutation. Founder effect can be a suggestive mechanism for the disease. Additionally, structural models of MSUD mutations have been performed to predict the pathogenesis of the newly identified variants.

**Keywords** MSUD · *BCKDHB* gene · Iran · Mutation analysis

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## Introduction

Maple Syrup Urine Disease (MSUD; OMIM #248,600) is a rare metabolic disease of branched-chain amino acid metabolism. Accumulation of leucine, isoleucine and valine and the corresponding branched-chain keto acids (BCKA) metabolites induce ketoacidosis, developmental disturbances, neurological impairment and coma and may be fatal if untreated (Korein et al. 1994; Yoshino et al. 1999). Therefore, early

detection and disease management are crucial for disease outcomes.

MSUD is a heterogeneous disorder with variable clinical severity. The disease is classified into five types based on the amount of branched-chain alpha-keto acid dehydrogenase (BCKD) activity. The first type is the classic which is the most severe with 0–2 % enzyme activity and neonatal presentation. The second is the intermediate form with 3–30 % enzyme activity and can present at any age. The third one is intermittent with 5–20 % activity of the enzyme and can present from infancy to adulthood. The fourth type is thiamin-responsive with 2–40 % activity and is the late onset form. The last type is dihydrolipoamide dehydrogenase deficiency ( $E_3$ -deficiency) that usually present during infancy. The enzyme activity is about 8–20 % (Shaw 2014).

The disease mainly results because of the impaired activity of BCKDC (BCKD; EC 1.2.4.4), the second enzyme in the catabolic pathway of branched chain amino acids. BCKDC is a member of the highly conserved mitochondrial  $\alpha$ -ketoacid dehydrogenase complexes. It is composed of three catalytic subunits: E1 (EC 1.2.4.4.), E2 (EC 2.3.1.168) and E3 (EC 1.8.1.4.) (Harris et al. 2004). The E1 subunit consists of a heterotetramer of  $\alpha_2\beta_2$  which functions as a BCKA dehydrogenase/decarboxylase (Chuang 2001).

The E1 $\beta$  subunit which is encoded by the *BCKDHB* gene is located at the 6q14 chromosomal position and includes 11 exons. It encodes for a 1.4 kb mRNA which is translated into 342 amino acid residues.

The E2 is a dihydrolipoyl transacylase that exists as a homo-24-mer and is the functional core of the complex (Chuang 2001). E3 is a dihydrolipoamide dehydrogenase and functions as a dihydrolipoyl dehydrogenase and is encoded by the *DBT* gene. Two regulatory enzymes (i.e. a branched-chain ketoacid dehydrogenase kinase (BCKDK) and protein phosphatase 2Cm (PPM1K or PP2Cm) regulate the activity of the complex (Lu et al. 2009; Reed et al. 1985). Defects in any part of the complex may lead to the disease state.

One of the considerable demographic features of the Iranian population is the elevated rate of consanguineous marriage. Unfortunately, the exact prevalence of MSUD is not clearly known in Iran, but it is expected to have a higher rate due to consanguineous marriage, than of the estimate of 1 in 185,000 affected infants (Chuang 2001) worldwide. This population structure is very suitable for homozygosity mapping studies because it increases the possibility of finding disease-causing mutations in blocks of homozygosity.

Genetic testing in MSUD is very helpful in Prenatal Diagnosis (PND) and Preimplantation Genetic Diagnosis (PGD) for at-risk families. It also facilitates molecular screening of patients to yield epidemiological data for implantation of community-based carrier testing in populations.

Homozygosity mapping was done with the help of STR (Short Tandem Repeats) markers to the above four mentioned genes to quickly identify the responsible gene in our MSUD patients.

The present survey aims to show a BCAA profile, and a clinical and molecular genetics study of families who showed homozygosity for the markers of in and around the *BCKDHB* gene. Haplotype analysis of four families with the same structure revealed the same mutation c.988G > A (p. Glu330Lys) (Fig. 1a). Haplotype B (Fig. 1b) was seen among three other families with a c. 508G > T (p. Arg170 Cys) mutation. Investigation of the remaining families revealed an insertion of 3 nucleotides.

c.833\_834insCAC (p. Gly278\_Thr279insThr), a splice site mutation c.633 + 1G > A and a homozygous deletion of exon-3. Computational analyzes were performed to see the impact of the novel mutations at the protein level.

## Material and methods

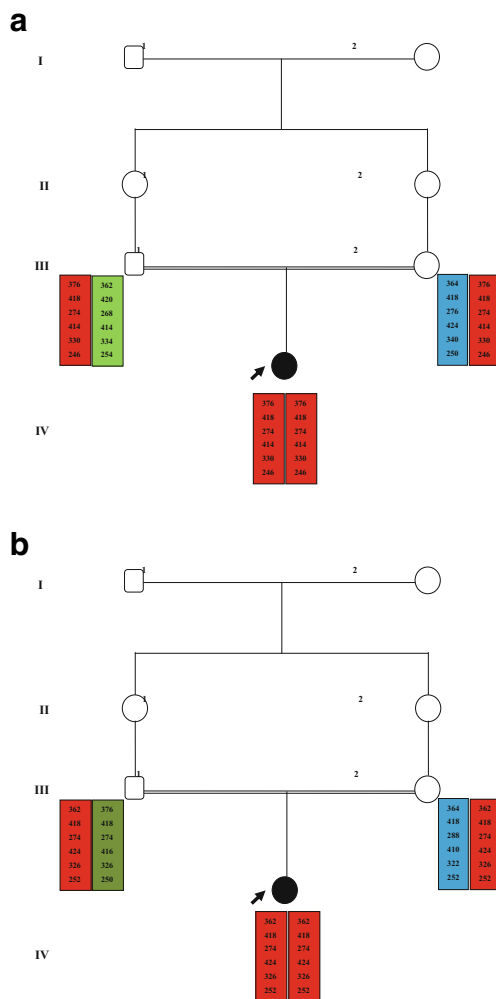
### Case descriptions

Twenty-one unrelated families were referred to our center by endocrine and metabolic specialists. The affected member of the family showed clinical presentations of MSUD and elevated levels of BCAAs and allo-isoleucine in the plasma and abnormal urine organic acids. Parents of all patients participating in this study had consanguinity. All patients studied here suffer from the classic form of the disease which is the most severe type. Genetic counseling was conducted and participants signed the consent form. The study was approved by the Ethical Committee of the Tehran University of Medical Sciences. Clinical features and the BCAA profile of each patient are presented in Table 1.

### Molecular genetic study

Five ml peripheral blood samples were taken from the patients and their parents in tubes containing EDTA as an anticoagulant. DNA was extracted from the blood samples by salting out method (Miller et al. 1988).

Suitable short tandem repeat (STR) markers flanking the *BCKDHA*, *BCKDHB*, *DBT* and *DLD* genes were selected using Tandem Repeat Finder (TRF) and Sequence-based Estimation of Repeat Variability (SERV) (Benson 1999; Legendre et al. 2007). An attempt was made to select markers with higher mean heterozygosity in the sample population. The other very important criterion in marker selection was the length of the repeats. Markers with 4–6 nucleotide repeats were preferred. We used six STR markers flanking each of the genes responsible for MSUD. These markers were mixed in two multiplex sets of 12 STRs. Primers for microsatellite



**Fig. 1** **a** The common haplotype structure is seen in the families with E330K mutation. **b** The common haplotype of the three families with R170 C mutation

markers were fluorescently labeled (Applied Biosystems) and primer sequences are available upon request. Fragment analysis was performed on an ABI 3130 Genetic Analyzer (Life Technologies, LT). The Genotypes of each individual were ascertained using Gene Mapper software. Then the haplotype for each family was drawn.

After finding the probably mutated gene, the entire coding region and intron-exon boundaries of the *BCKDHB* gene were amplified from genomic DNA. Primers are available upon request. PCR amplification was performed based on the protocol reported previously (Foroozani et al. 2015).

PCR products were directly sequenced using BigDye Terminator kit (Thermo Fisher Scientific, Life Technologies, USA, TS) according to the manufacturer's protocol. The samples were run on an ABI3130XL Genetic Analyzer at the KBC (Kawsar Biotech Company) facility. Sequences were compared with human genomic and cDNA sequences of the *BCKDHB* gene with the accession number (NC\_000006.11, NM\_183050.2).

Nomenclature of the novel mutations was performed according to the recommendations of Human Genome Variation Society (Den Dunnen and Antonarakis 2000).

In the case of novel missense mutations, Amplification Refractory Mutation System (ARMS) was performed on 50 healthy subjects from the same ethnicity to check the presence of mutant or normal form of the allele.

### Computational analysis

The X-ray crystal structure of E1b (PDB entry: 2J9F) was used as the initial conformation for computational analysis. Modeller 9.13 was used for repairing missing residues (Eswar et al. 2006). Generation of p. Gly278\_Thr279insThr mutant structure was performed with Modeller 9.13 (Eswar et al. 2006). Hydrogen was added using reduce 3.23 for the mutant of p. Glu330Lys, p. Arg170Cys, p. Gly278\_Thr279insThr and the wild-type complexes. Then, structures were prepared for energy minimization using the psfgen plugin of VMD (Humphrey et al. 1996).

All complex systems were minimized to 1000 steps by the conjugate gradient algorithm using NAMD software package to remove bad contacts (Phillips et al. 2005). The hydrogen bonds and salt bridges were measured by H Bonds and Salt Bridges plugin of VMD, respectively.

Structural impact of exon-3 deletion on *BCKDHB* was analyzed by generating the mutant conformation from the crystal structure (PDB entry: 2J9F) by I-TASSER web server (Roy et al. 2010; Yang et al. 2015; Yang and Zhang 2015; Zhang 2008). The Best proposed model was selected based on C-score and estimated TM-score for further analysis. Then, the wild type and mutant complexes were superimposed and secondary structure distributions were assessed by Timeline plugin of VMD (Humphrey et al. 1996). VMD was used for creating molecular graphics (Humphrey et al. 1996). Further analysis was accomplished by R.

### Results

The study included twenty-one families with clinical diagnosis of MSUD. Ten patients showed homozygous haplotypes for markers flanking the *BCKDHB* gene, six for *BCKDHA*, two for *DBT* and no family was homozygous for the markers flanking the *DLD* gene. The remaining three families did not show homozygosity to any of the mentioned genes. All ten patients with homozygous haplotype block of the *BCKDHB* gene had the classic form of the disease which is the most severe form.

Investigation of all exons and exon/intron boundaries of the *BCKDHB* gene allowed successful identification of the disease-causing mutations in the investigated families. The identified mutations in the patients were homozygous as

**Table 1** Genotype, clinical features and biochemical profile of each studied patient

Patient ID	Sex	Genotype	Amino acid change	Haplotype	Age at diagnosis	Consanguinity of parents	Family history	Clinical symptoms	Current age of the patient	GC-MS organic acids	BCAA at diagnosis ( $\mu\text{mol/L}$ )**
P1	M	c.[988G > A] + [988G > A]	p.[Glu330]ys] + [Glu330]ys]	A	4 M	yes	No	Seizures, severe DD, maple syrup urine odor	8Y	MSUD pattern*	Leu + Ile:1143 (Leu + Ile:1143)/phe:24 Val:207
P2	M	c.[988G > A] + [988G > A]	p.[Glu330]ys] + [Glu330]ys]	A	25 D	yes	Yes	Hypotonia, poor feeding, maple syrup urine odor	Died at 2Y,5 M	MSUD pattern	Leu + Ile:2543 (Leu + Ile:1143)/phe:40 Val:131
P3	M	c.[988G > A] + [988G > A]	p.[Glu330]ys] + [Glu330]ys]	A	9 D	Yes	No	Seizures, poor feeding, Sever DD	2Y,4 M	MSUD pattern	Ile:382.5 Leu:1158 Val:322.6
P4	F	c.[988G > A] + [988G > A]	p.[Glu330]ys] + [Glu330]ys]	A	14 D	Yes	No	Poor feeding, hypotonia	Died at 1 M	MSUD pattern	Leu + Ile:2723 (Leu + Ile:1143)/phe:65
P5	M	c.[508G > T] + [508G > T]	p.[Arg170Cys] + [Arg170Cys]	B	9D	Yes	No	Seizures, maple syrup urine odor, irritability	Died at 17 D	MSUD pattern	Val: not elevated Ile:829 Leu:3453 Val:709
P6	F	c.[508G > T] + [508G > T]	p.[Arg170Cys] + [Arg170Cys]	B	4 M	Yes	NO	Poor feeding, Seizures	Died	MSUD pattern	Leu + Ile:3492 (Leu + Ile:1143)/phe:123 Val:232
P7	M	c.[508G > T] + [508G > T]	p.[Arg170Cys] + [Arg170Cys]	B	9D	Yes	No	Lethargy, maple syrup urine odor, hypotonia	Died at 70 D	MSUD pattern	Leu + Ile:3460 (Leu + Ile:1143)/phe:102 Val:289
P8	M	c.[633 + 1G > A] + [633 + 1G > A]	-	-	1 M	Yes	No	Sever DD, seizures, poor feeding, lethargy	2Y,4 M	MSUD pattern	Ile:589 Leu:2444 Val:472
P9	M	c.833_834insCAC	p. Gly278_Thr279msThr	-	19D	Yes	No	Dead, Sever DD	Died at 2Y,5 M	MSUD pattern	Ile:542 Leu:2932 Val:397
P10	F	c.(274 $\pm$ 1_275-1)_ (343 $\pm$ 1_344-1)	-	-	20D	Yes	No	Maple syrup urine odor, seizures, Sever DD	14Y	MSUD pattern	Ile:385 Leu:1173 Val:683

M Month, D Day, DD Developmental Delay

\*MSUD pattern here means -increased levels of ketoacids; 2-OH isovalerate, 2-OH isocaproate, 2-OH-3- methylvalerate, 2-keto-3-methylval erate, 2-ketoisocaproate

\*\*Leucine +Isoleucine cut off <350  $\mu\text{mol/L}$ , Leu + Isoleu/phe cut off < 10  $\mu\text{mol/L}$ , Val cut off < 250  $\mu\text{mol/L}$

expected by the homozygote haplotype results. Parents were heterozygous for the identified mutations (Table 1).

Analysis of ten families with the homozygote haplotype of the *BCKDHB* gene revealed four families with the same haplotype structure (Fig. 1a) and further analysis showed the same mutation (c.988G > A). Three of the other families showed the B haplotype with the same mutation of c.508G > T (Fig. 1b). The last three families showed a different haplotype and also a different mutation including a splice site mutation (c.633 + 1G > A), an insertion (C.833\_834insCAC) and homozygous deletion of exon-3 of the gene c. (274 + 1\_275–1)\_(343 + 1\_344–1).

The relative locations of the mutations of p. Gly278\_Thr279insThr, p. Glu330Lys and Arg170Cys in E1b heterodimer are shown in Fig. 2. As shown in this figure, Gly278\_Thr279insThr and p. Glu330Lys mutations are located near the end of the alpha helix and beta sheets secondary structures of the  $\beta$  subunit of the BCKDC. The p. Arg170Cys mutation is located at the interface of the  $\beta$ ,  $\beta'$  and  $\alpha$  subunits close to the metal ion and cofactor binding site. To elucidate the individual structural impact of these mutations on conformational stability of E1b, hydrogen bonds, and salt bridges were calculated in mutant complexes compared to the wild type after energy minimization. All the mutations resulted in redistribution of hydrogen bonds, mostly in the  $\beta$  and  $\alpha$  subunits (Figs. 3 and 4).

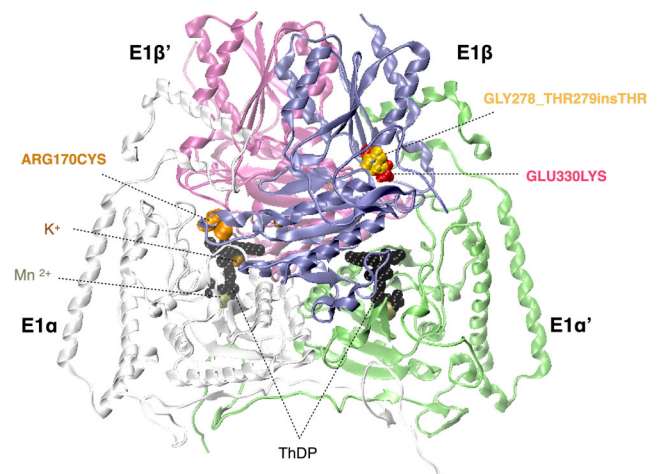
Figure 3a, b display the exclusive hydrogen bonds and salt bridges in wild-type and p. Arg170Cys mutant conformations, respectively. As shown in Fig. 3, the p. Arg170Cys mutation affected not only the intra-subunit, but also inter-subunit interactions between  $\alpha$  and  $\beta$  subunits. In particular, substitution of Arginine by Cysteine resulted in the elimination of salt bridges (green dash lines) between Arg170 and Glu163 inside the  $\beta$  subunit. Moreover, the hydrogen bond between Ser206- $\alpha$  and Glu163- $\beta$  was also removed. The rearrangement of these bonds might have an influence on the stability of adjacent loops due to the close distance to the potassium ion. This situation might also affect the ThDP (Thiamin Diphosphate) binding site.

The superimposition of wild type and mutant complexes with their exclusive hydrogen bonds is shown in Figs. 4a, b, respectively. As shown in these figures, insertion of Threonine between Glycine 278 and Threonine 279 results in the alteration of loop structure between sheet A and the following helix. This insertion retreated sheets B and C in comparison to the wild type (Fig. 4a, b). The mutation is involved in the redistribution of adjacent amino acids near the  $\beta$ - $\beta'$  interfaces (e.g. Glu330, Ala331, Pro332 in wild-type and Glu331, Ala332, Pro333 in the mutant). These changes have an important role in alteration of local hydrogen bonds (i.e. elimination of hydrogen bonds between Glu201 and Trp277 in the wild-type and formation of new hydrogen bonds between Thr279 and Glu331 in the mutant structure).

As shown in Figs. 5a, b, mutation of Glutamate to Lysine at the position of 330 induced rearrangements of some local hydrogen bonds including the elimination of Glu330-Cys188. This mutation is located near the subunit interface of  $\beta$ - $\beta'$ .

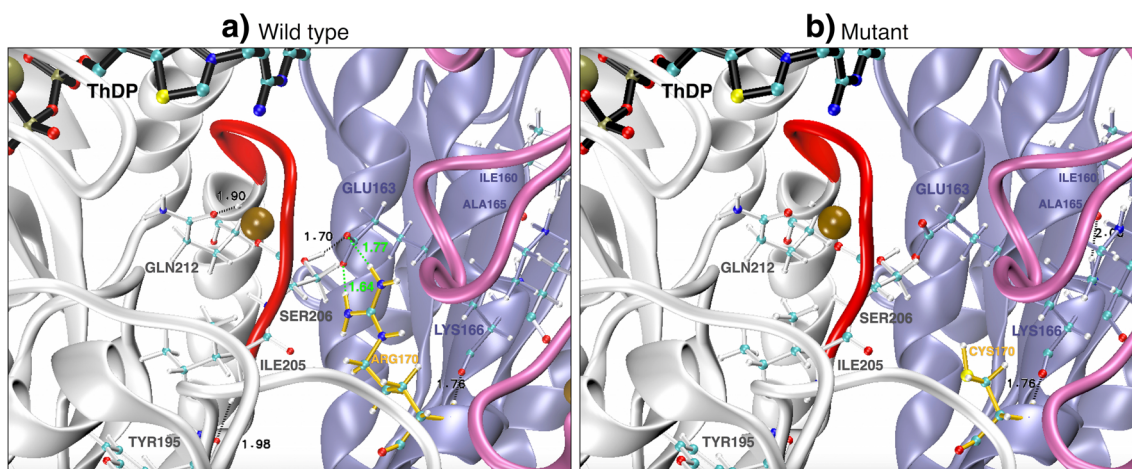
Failure to amplify genomic DNA covering the third exon of the *BCKDHB* gene indicated the possibility of the presence of a deletion or at least a SNP (single nucleotide polymorphism) in one of the primer annealing sites. Changing the primer sequences or its location did not solve the problem, therefore we designed a duplex PCR to simultaneously amplify the first and third exons of the gene for the patient. Since in a reaction of duplex PCR the internal control primers gave a bond and the third exon did not give its relative bond, it could be concluded that there was a deletion that covers at least one of the primer annealing sites. In addition, we tried to amplify the genomic DNA using the forward primer of exon 2 and the reverse primer for exon 3. The result was no amplification. We also tried the forward primer of exon 3 and the reverse primer of exon 4, yet no band was produced, indicating complete deletion of exon 3.

Further analysis of the exon-3 deletion was completed using I-TASSER to investigate the impact on protein structure. The model with C-score 1.91 and estimated TM-score  $0.99 \pm 0.04$  was selected. The RMSD value between experimentally determined structure and predicted model was estimated as  $2.5 \pm 1.9$  Å. As shown in Fig. 6, the predicted model and crystal structure were structurally compared after the superimposition. These results revealed that exon-3 deletion removes 25 residues within the protein sequence, ranging from Lys92 to Gly116 (Fig. 6b). Therefore, the mutation led



**Fig. 2** a 3D structural representation of the E1b heterotetramer: E1 $\alpha$  (white); E1 $\beta$  (blue); E1 $\alpha'$  (green) and E1 $\beta'$  (pink). The mutated residues Arg170Cys, Gly278\_Thr279insThr and Glu330Lys are indicated in orange, yellow and red, respectively. Magnesium and potassium ions are presented in light and dark brown, respectively. Thiamin diphosphates (ThDPs) are shown in black. For detailed description see “Results and Discussion”





**Fig. 3** The structural illustration of the mutated region related to the Arg170Cys mutation. **a** Arginine 170 situation with exclusive hydrogen bonds and salt bridges in the wild-type conformation. **b** Cysteine 170 position with exclusive hydrogen bonds in the mutant conformation. Red loop corresponds to the residues of 206 to 211 of the E1 $\alpha$  subunit. Mutated residue is colored yellow. E1 $\alpha$ , E1 $\beta$ , E1 $\alpha'$ , and E1 $\beta'$  are shown

to the elimination of the two  $\beta$ -sheets at the interface of the  $\beta/\beta'$  with  $\alpha/\alpha'$  near the ligand binding sites (Fig. 6a).

## Discussion

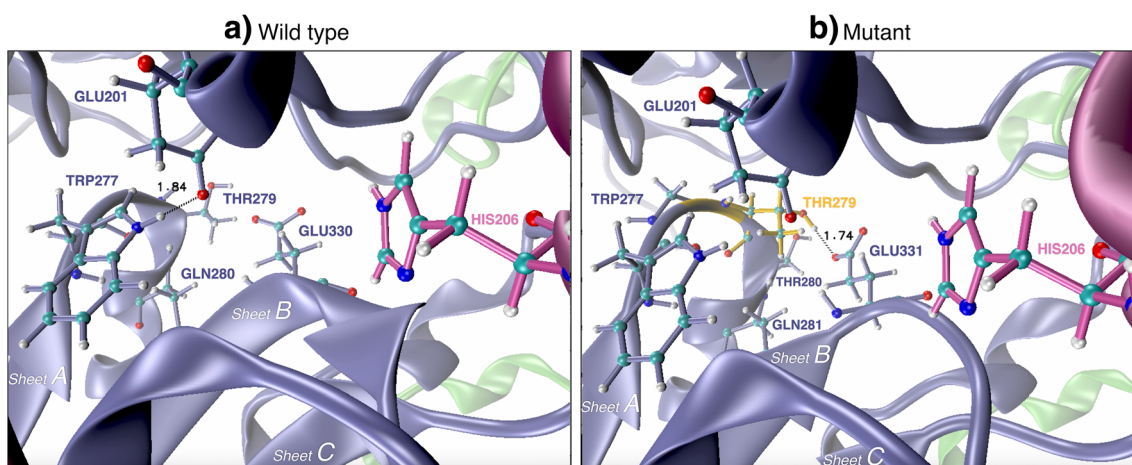
Homozygosity mapping is an efficient tool for identifying genetic defects underlying genetically heterogeneous disorders such as inborn errors of metabolism. This tool is especially useful in populations with a high rate of consanguineous marriages.

Haplotype analysis of the studied families showed ten families with homozygous haplotype block for the *BCKDHB* gene, six for the *BCKDHA*, two for the *DBT* and no family

was homozygous for the markers flanking the *DLD* gene. Subsequently, we sequenced the corresponding gene in each family.

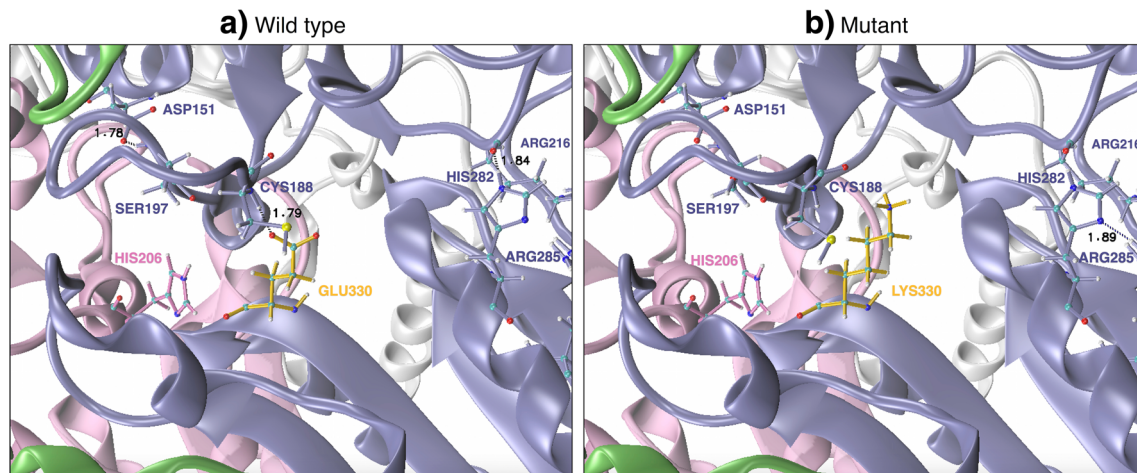
Iran is a country with a consanguineous marriage rate of approximately 38 % (Saadat et al. 2004), so naturally the rate of autosomal recessive disorders will be higher in comparison to the Western countries. The rarer a disease, the higher the probability it will occur in a consanguineous marriage. MSUD is a rather rare disease even in Iran with a high rate of consanguineous marriage. Very few studies have been published regarding mutation frequencies in Iran (Miryounesi et al. 2015). With this in mind, we conducted homozygosity mapping for twenty-one families with MSUD.

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**Fig. 4** The structural illustration of the mutated region related to the Gly278\_Thr279insThr mutation. **a** The position of the mutation before the insertion of Threonine with exclusive hydrogen bonds in the wild-type conformation. **b** The location of the inserted Threonine between Gly278 and Thr279 with exclusive hydrogen bonds in the mutant

conformation. Mutated residue is colored yellow. E1 $\beta$ , E1 $\alpha'$ , and E1 $\beta'$  are shown in blue, green and pink, respectively. Oxygen, nitrogen, sulfur and carbon atoms are in red, blue, yellow and cyan, respectively. Hydrogen bonds are indicated by black dash-lines. For detailed descriptions see “Results and Discussion”



**Fig. 5** The structural illustration of the mutated region related to the Glu330Lys mutation **a** Glutamate 330 situation with exclusive hydrogen bonds in the wild-type conformation. **b** Lysine 330 position with exclusive hydrogen bonds in the mutant conformation. The mutated residue is colored yellow. E1 $\alpha$ , E1 $\beta$ , E1 $\alpha'$ , and E1 $\beta'$  are

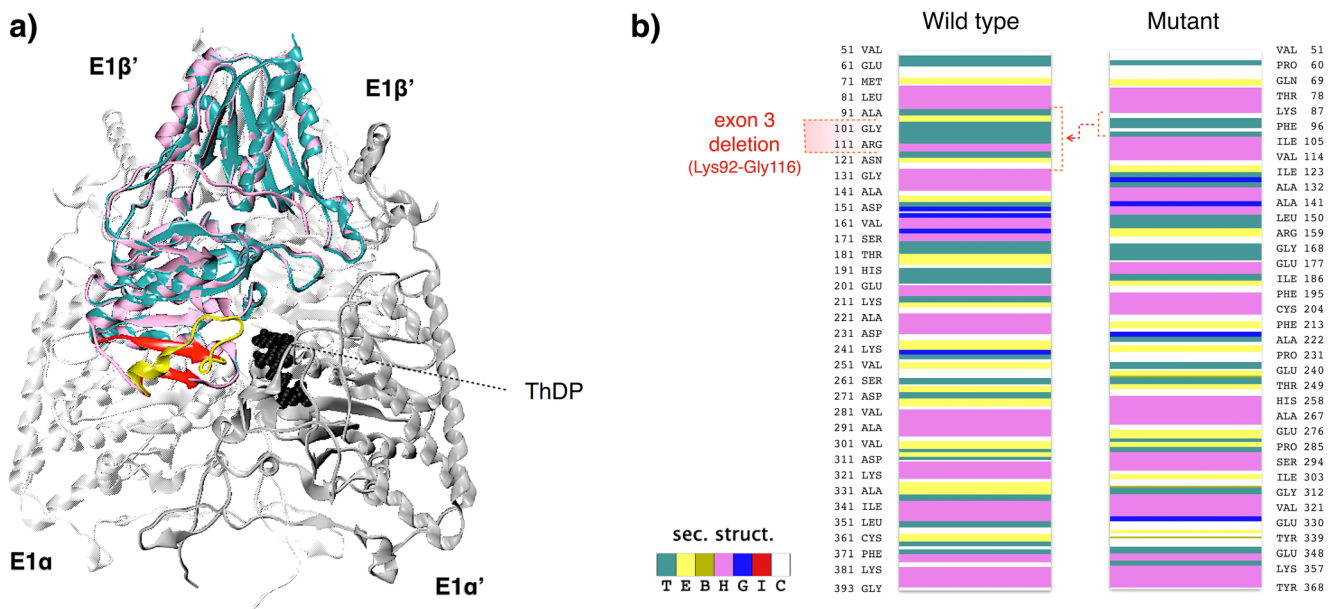
shown as white, blue, green and pink, respectively. Oxygen, nitrogen, sulfur and carbon atoms are in red, blue, yellow and cyan, respectively. Hydrogen bonds are indicated by black dash-lines. For a detailed description see “Results and Discussion”

A very important finding was an observation of two common haplotypes among investigated families (Fig. 1a, b). Haplotype A was seen in four families. To determine whether these families are related or from the same ancestral origin, we interviewed the families and asked about their ethnicity. Our analysis showed the families are of Lak origin which lives in Lorestan province. Such findings in a country with high genetic heterogeneity and, on the other hand, disease rarity of MSUD brought us to the conclusion that founder effect may play a strong role in this situation. The four patients with this

mutation either died or suffer from severe developmental delay. The three patients with the B haplotype carrying same mutation died before the age of one month. Of these three patients, two patients belong to the Lurs and one is of Kurdish origin.

Mutations discovered in this study are categorized into two groups based on their structural-functional impacts on the E1b complex.

The first category includes mutations that might directly or indirectly affect subunit interfaces i.e. the p. Arg170Cys



**Fig. 6** The 3D structural representation of exon-3 deletion in the *BCKDHB* gene. **a** The wild-type (cyan) and predicted mutant E1 $\beta'$  (pink) are shown within the E1b complex; the mutated part, deleted residues within the sequence of Lys92 to Gly116, is indicated in yellow; removed regions in the wild-type structure are presented in red.

**b** The secondary structure of the wild-type and mutant conformations. Missing regions including two  $\beta$ -parallel sheets are indicated by the red arrow. T: Turn, E: Extended conformation ( $\beta$ -sheets), B: Isolated bridge, H:  $\alpha$ -helix, G: 3–10 helix, I: Pi-pihelix, C: Coil. For a detailed description see “Results and Discussion”

**Table 2** prediction of pathogenicity of novel mutations

Mutation	Exone	Protein	SIFT prediction	Polyphen prediction	Mutation Taster prediction
c.633 + 1G > A	5	–	–	–	Disease causing:
C.833_834insCAC	7	p. Gly278_Thr279insThr	–	–	Disease causing:
c.988G > A	9	p. Glu330Lys	Probably damaging	Damaging	Disease causing:
c. 508G > T	5	p. Arg170 Cys	Probably damaging	Damaging	Disease causing:

which is directly in contact with  $\alpha$ ,  $\beta$  and  $\beta'$  interfaces, and p. Glu330Lys and p. Gly278\_Thr279insThr which might affect the local residues adjacent to the interface of the  $\beta$ – $\beta'$  subunits. The second category consists of mutations that might have an influence on the metal ion interaction and also ThDP cofactor binding (p. Arg170Cys) (Figs. 2 and 3).

Ævarsson et al. demonstrated that the specific conserved loop located between residues 206 and 211 of the  $\alpha$  subunit is stabilized by the potassium ion interactions. This interaction is also critical for the enzyme's activity due to the contribution of this loop to cofactor binding (ThDP) (Ævarsson et al. 2000). Our analysis indicated that the p. Arg170Cys mutation resulted in the breakage of salt bridges between Arg170– $\beta$  and Glu163– $\beta$  which subsequently affect the interactions between Ser206– $\alpha$ –Glu163– $\beta$  and Ser206– $\alpha$ –Gln212– $\alpha$ . Due to the critical role of Ser206– $\alpha$  in the aforementioned loop, this mutation has an influence on both the structure and function of the E1b complex (Fig. 3a, b). Moreover, the proposed assembly pathway of E1b indicates that the tetrameric assembly process is composed of two steps: 1) The heterodimerization of  $\alpha/\beta$  and  $\alpha'/\beta'$ , and 2) Subsequent heterotetramerization of  $\alpha/\beta$ – $\alpha'/\beta'$  (Ævarsson et al. 2000). Since the p. Arg170Cys mutation is placed at the  $\alpha$ – $\beta$  subunit interfaces, this might destabilize multimerization of the whole structure during complex assembly (Figs. 1 and 2).

As earlier studies showed the essential role of the residues which are located at the interface of the  $\beta$ – $\beta'$  subunits of E1b including His206 from the  $\beta'$  and Trp277, Pro332, Tyr334, Glu201 and Phe203 from the  $\beta$  subunit (Ævarsson et al. 2000), the insertion of Threonine between Gly278 and Thr279 not only led to conformational changes in the following two extended sheets (sheets B and C) near this position, but also opened the loop structure between sheet A and the subsequent helix (Fig. 4). These alterations caused loss of hydrogen bonds between Glu201– $\beta$  and Trp277– $\beta$  and the formation of new hydrogen bonds between Thr279– $\beta$  and Glu331– $\beta$  (Fig. 4a, b). Due to the redistribution of the local residues of sheet A, followed by the helix, sheet B, and C, which are adjacent to the His206 of  $\beta'$  subunit, this mutation might effect on the association of two  $\beta$  subunits.

Regarding the substitution of lysine for glutamic acid (E330K) (Fig. 2), substitution of negatively charged glutamate with positively charged lysine not only affected the local electrostatic interactions, but also the hydrogen bonds (Fig. 5). This

mutation is close to the two  $\beta$  subunits which led to the rearrangement of inter-chain hydrogen bonds in the  $\beta$  subunit including the elimination of the Glu330– $\beta$ –Cys188– $\beta$  hydrogen bonds (Fig. 5a). Due to the close distance of this mutation to the  $\beta$ – $\beta'$  subunits, this might have an influence on E1b stability.

The structural effect of exon-3 deletion of the *BCKDHB* gene on protein structure was evaluated after homology modeling (Fig. 6). The deletion resulted in the elimination of residues from Lys92 to Gly116. Elimination of the mentioned residues resulted in the removal of two parallel sheets within the interface of  $\alpha/\beta$  subunits. This might have strong negative influence not only in the assembly of the E1b complex, but also on the binding of ThDP nearby. Although the diagnosis of the disease was at 20 days, diet and medication were not effective on her disease recovery.

To further evaluate the pathogenicity of the identified variants, we checked for the presence of the identified changes in 50 healthy unrelated individuals from the same ethnic backgrounds. None of them showed the mentioned changes. Also, none of the identified variants are present in the single nucleotide polymorphism database (Exome Variant Server, <http://evs.gs.washington.edu/EVS/>). These predictions are also supported by bioinformatics predictions by Polyphen-2 (Adzhubei et al. 2010) which indicates that this is “probably damaging” and SIFT (Kumar et al. 2009) indicated that these mutations are “damaging” (Table 2).

To understand the effect of the mutation on the one patient with the insertion of three nucleotides (c.833\_834ins3), which added a threonine to the protein structure, Human Splice Finder (HSF) (Desmet et al. 2009) was utilized. This program predicted that the insertion will alter the wild-type donor splice site and probably affect splicing. HSF prediction algorithms predict that it decreases the consensus value of wild type from 82.65 to 11.1 for the mutant. Also, the predicted variation score is –86.57 % (this prediction is much lower than the significance level of –10 % in HSF).

To establish genotype-phenotype correlations, more cases need to be studied. These findings will add to our molecular understanding of MSUD in Iran and other countries.

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**Compliance with ethical standards**



**Conflict of interest** The authors declare no conflict of interest.

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