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ORIGINAL ARTICLE

Maple syrup urine disease mutation spectrum in a cohort of 40 consanguineous patients and insilico analysis of novel mutations

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Abstract

Maple syrup urine disease is the primary aminoacidopathy affecting branched-chain amino acid (BCAA) metabolism. The disease is mainly caused by the deficiency of an enzyme named branched-chained α-keto acid dehydrogenase (BCKD), which consist of four subunits (E1α, E1β, E2, and E3), and encoded by *BCKDHA, BCKDHB, DBT*, and *DLD* gene respectively. BCKD is the main enzyme in the catabolism pathway of BCAAs. Hight rate of autosomal recessive disorders is expected from consanguineous populations like Iran. In this study, we selected two sets of STR markers linked to the four genes, that mutation in which can result in MSUD disease. The patients who had a homozygous haplotype for selected markers of the genes were sequenced. In current survey, we summarized our recent molecular genetic findings to illustrate the mutation spectrum of MSUD in our country. Ten novel mutations including c.484 A > G, c.834_836dup CAC, c.357del T, and c. (343 + 1_344–1) _ (742 + 1_743–1)del in *BCKDHB*, c.355–356 ins 7 nt ACAAGGA, and c.703del T in *BCKDHA*, and c.363delCT/c.1238 T > C, c. (433 + 1_434–1) _ (939 + 1_940–1)del, c.1174 A > C, and c.85_86ins AACG have been found in DBT gene. Additionally, structural models of MSUD mutations have been performed to predict the pathogenicity of the newly identified variants.

Keywords MSUD · Amino acid metabolism · Mutation · Consanguinity · Iran

Introduction

Maple syrup urine disease (MSUD; OMIM #248,600) is the primary aminoacidopathy affecting branched-chain amino acid metabolism. MSUD patients can present in the infancy with ketoacidosis, hyperammonemia, altered level of consciousness, neurological impairment, coma, and basal ganglia injury, and may be fatal if remain untreated (Burrage et al. 2014; Miller et al. 1988; Zeltner et al. 2014). Therefore, early diagnosis and disease

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management are preeminent for disease outcome (Yoshino et al. 1999).

There is a continuum of clinical heterogeneity for MSUD. Residual enzyme activity of the dehydrogenase complex influence the severity of the disease. There are four types of the disease based on the clinical manifestations. First, is the classic form with 0–2% *BCKD* enzyme activity which presents in neonates. The second form is intermediate which present from infancy to adulthood with 3–10% enzyme activity. The third from is thiamine-responsive, which has late-onset presentation and *BCKD* enzyme activity is about 2–40%. Dihydrolipoamide dehydrogenase efficiency is the last form with 8–20% enzyme activity and infancy presentation (Shaw 2014).

The branched-chain amino acid dehydrogenase enzyme complex comprised of four critical subunits, which decarboxylates, leucine, isoleucine, and valine into branched-chain organic acids. Clinical manifestations of the disease is accompanied by accumulation of branched-chain amino acids in body fluid as a result of the genetic mutations in that subunits (Burrage et al. 2014).

Branched-chain keto acid dehydrogenase (BCKD) is a large multienzyme complex and is located on the outer face of the inner mitochondrial membrane. It consists of three catalytic subunits: E1 (EC 1.2.4.4.), included two separate proteins: E1α and E1β, which forms a decarboxylase, E2 (EC 2.3.1.168), which consist of a protein named transacylase, and E3 (EC 1.8.1.4.), that included a protein named lipoamide oxidoreductase (Blackburn et al. 2017; Harris et al. 2004). The E1α which is encoded by the *BCKDHA* gene is located at the 19q13. The E1β is encoded by the *BCKDHB* gene located at 6q14. E2 exists as a homo-24-mer and is the functional core of the complex. It encodes by *DBT* gene that is located at the 1p21. E3 subunit is encoded by the *DLD* gene located at 7q31.

Due to high rate of consanguineous marriage in Iran, it is expected to have higher rate of autosomal resessive disease prevalence, than of the estimated rate in other populations. Populations with high rate of consanguineous marriage is very suitable for homozygosity mapping studies. In such inbreed populations the chance of finding disease-causing mutations in blocks of homozygosity may be increase. Homozygosity mapping was conducted with the help of STR markers linked to the four mentioned genes to identify the responsible mutated gene in our MSUD patients. Identification of new variants and their functional explanation in MSUD facilitates prenatal diagnosis (PND) as well as a pre-implantation genetic diagnosis (PGD). It may also facilitate the implementation of community-based carrier testing in the population.

The aim of the present study was to investigate the mutation spectrum of MSUD patients in the consanguineous population of Iran. Homozygosity mapping method was performed to identify the gene responsible in our MSUD patients. The markers were selected based on the heterozygosity in the studied population. Computational analyses were performed to see the impact of novel mutations at the protein level.

Material and methods

Patients' presentations

The samples were collected from forty unrelated families were referred to our center by metabolic disorder specialists. Inclusion criteria include clinical presentations of the disease and abnormal urine organic acids and elevated levels of BCAAs and allo-isoleucine in the plasma. Written consent form for participation, after genetic counseling, was obtained. The research ethics committee of the Tehran University of Medical Sciences approved the study. Table 1 shows Clinical and BCAA information for all patients. Figures 1 illustrate the mutation distribution of each genes, as well as the location of mutations and the severity of the disorder.

Genetic testing

Five ml peripheral blood samples with EDTA anticoagulant were collected from each patient, parents. A salting out procedure was used to extract Genomic DNA from peripheral blood samples (Miller et al. 1988). For indirect mutation analysis and identification possible causative gene, homozygosity mapping was performed using six polymorphic STR markers flanking each of MSUD genes (the *BCKDHA, BCKDHB, DBT,* and *DLD*). Appropriate short tandem repeat (STR) markers were selected using, Tandem Repeat Finder (TRF) and Sequence-based Estimation of Repeat Variability (SERV) (Benson 1999).

The criteria of selected markers were the high level of polymorphism (informativeness) in the same population, length of the repeats (4–6 nucleotide repeats) and the distance and proximity of the markers with the gene. The flanking markers used in two multiplex sets consisting of 12 STR markers. The Fluorescently labeled Primers (Applied Biosystems) (Location and primer sequences for each marker of MSUD genes are available upon request) flanking STR markers were used in a multiplex PCR and then the fragment analysis was done on an ABI 3130 Genetic Analyzer (Life Technologies, LT). Gene Mapper software was used for analyzing Genotyping of each individual and then haplotype drawing was done for each family.

Haplotype blocks were mapped from the candidate markers of the mentioned genes, consequently, mutation analysis was performed using specific primers that amplify all exons and flanking sequences. The exon amplification and sequencing were performed in 25 reactions for all samples. Concisely, 1 U Taq DNA polymerase

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

Patient ID	Sex	Genotype	Amino acid change	Gene	Age at diagnosis	Consanguinity Of parents	Clinical symptoms	GC-MS organic acids*	BCAA at diagnosis (μmol/L)**	References
P1	M	c.[988G > A] + [988G > A]	p.[Glu330lys] + [Glu330lys]	BCKDHB	4 M	Yes	Seizures, severe DD, maple syrup urine odor	MSUD pattern	Leu + Ile:1143 (Leu + Ile:1143)/phe:24 Val:207	(Abiri et al. 2017)
P2	M	c.[988G > A] + [988G > A]	p.[Glu330lys] + [Glu330lys]	BCKDHB	25 D	Yes	Hypotonia, poor feeding, maple syrup urine odor	MSUD pattern	Leu + Ile:2543 (Leu + Ile:1143)/phe:40 Val:131	(Abiri et al. 2017)
P3	M	c.[988G > A] + [988G > A]	p.[Glu330lys] + [Glu330lys]	BCKDHB	9 D	Yes	Seizures, poor feeding, Sever DD	MSUD pattern	Ile:382.5 Leu:1158 Val:322.6	(Abiri et al. 2017)
P4	F	c.[988G > A] + [988G > A]	p.[Glu330lys] + [Glu330lys]	BCKDHB	14 D	Yes	Poor feeding, hypotonia	MSUD pattern	Leu + Ile:2723 (Leu + Ile:1143)/phe:65 Val: not elevated	(Abiri et al. 2017)
P5	M	c.[508G > T] + [508G > T]	p.[Arg170Cys] + [Arg170Cys]	BCKDHB	9 D	Yes	Seizures, maple syrup urine odor, irritability	MSUD pattern	Ile:829 Leu:3453 Val:709	(Abiri et al. 2017)
P6	F	c.[508G > T] + [508G > T]	p.[Arg170Cys] + [Arg170Cys]	BCKDHB	4 M	Yes	Poor feeding, Seizures	MSUD pattern	Leu + Ile:3492 (Leu + Ile:1143)/phe:123 Val:232	(Abiri et al. 2017)
P7	M	c.[508G > T] + [508G > T]	p.[Arg170Cys] + [Arg170Cys]	BCKDHB	9 D	Yes	Lethargy, maple syrup urine odor, hypotonia	MSUD pattern	Leu + Ile:3460 (Leu + Ile:1143)/phe:102 Val:289	(Abiri et al. 2017)
P8	M	c.[633 + 1G > A] + [633 + 1G > A]	_	BCKDHB	1 M	Yes	Sever DD, seizures, poor feeding, lethargy	MSUD pattern	Ile:589 Leu:2444 Val:472	(Abiri et al. 2017)
P9	M	c.[833_834insCAC]	p. Gly278_Thr279insThr	BCKDHB	19 D	Yes	Dead, Sever DD	MSUD pattern	Ile:542 Leu:2932 Val:397	(Abiri et al. 2017)
P10	F	c.(274 ± 1_275–1)_(343 ± 1_344–1)del	_	BCKDHB	20 D	Yes	Maple syrup urine odor, seizures, Sever DD	MSUD pattern	Ile:385 Leu:1173 Val:683	(Abiri et al. 2017)
P11	F	c.[853C > T] + [853C > T]	p.[Arg285X] + [Arg285X]	BCKDHB	3 D	Yes	Hypotonia, poor feeding, maple syrup urine odor	MSUD pattern	Val:395 Leu + Isoleu/phe:108 Leu + Isoleu:4003	(Henneke et al. 2003)
P12	M	c.[834_836dup CAC]	_	BCKDHB	32 D	Yes	Sever DD, seizures, poor feeding, lethargy, maple syrup urine odor	MSUD pattern	Val:596 Leu + Isoleu/phe:108 Leu + Isoleu:4003	Novel
P13	F	c.[599C > T] + [599C > T]	p.[Pro200Leu] + [Pro200Leu]	BCKDHB	8 D	Yes	Maple syrup urine odor, seizures, Sever DD	MSUD pattern	Leu: 530.7 Isoleu:129.3	(Narayanan et al. 2013)
P14	F	c.[730 T > C] + [730 T > C]	p.[Tyr244His] + [Tyr244His]	BCKDHB	1 M	Yes	Poor feeding, seizure, irritability, maple syrup urine odor	MSUD pattern	Leu: 460 Isoleu:109.1	(Flaschker et al. 2007)
P15	M	c.[508C > T] + [508C > T]	p.[Arg170Cys] + [Arg170Cys]	BCKDHB	22 D	Yes	Sever DD, seizures, poor feeding, lethargy, maple syrup urine odor	MSUD pattern	Val: 38 Isoleu:118 Leu: 824	(Jung Min et al. 2014)
P16	F	c.[410C > T] + [410C > T]	p.[Ala137Val] + [Ala137Val]	BCKDHB	20 D	Yes	Poor feeding, seizure, irritability	MSUD pattern	Val:683.8 Isoleu:385.4 Leu:1173	(Nellis et al. 2003)

Table 1 (continued)

Patient ID	Sex	Genotype	Amino acid change	Gene	Age at diagnosis	Consanguinity Of parents	Clinical symptoms	GC-MS organic acids*	BCAA at diagnosis (μmol/L)**	References
P17	M	c.[508C > T] + [508C > T]	p.[Arg170Cys] + [Arg170Cys]	BCKDHB	7 D	Yes	Poor feeding, seizure, irritability, maple syrup urine odor	MSUD pattern	Val:433 Isoleu:193 Leu:691	(Jung Min et al. 2014)
P18	M	c.[988G > A] + [988G > A]	p.[Glu330Lys] + [Glu330Lys]	BCKDHB	25 D	No	Poor feeding, seizure, irritability, maple syrup urine odor	MSUD pattern	Val:333.3 Leu:1471.9 Leu:1471.9	(Safdarian et al. 2016)
P19	F	c.(343 + 1_ 344–1)_ (742 + 1_ 743–1)del	_	BCKDHB	7 D	Yes	Sever DD, seizures, poor feeding, lethargy, maple syrup urine odor	MSUD pattern	Val:391 Isoleu:366 Leu:1777	Novel
P20	F	c.[484 A > G] + [484 A > G]	p.[Asn162Asp] + [Asn162Asp]	BCKDHB	5 D	Yes	Poor feeding, seizure, irritability, maple syrup urine odor	MSUD pattern	Val:311 Leu + Isoleu/phe:108 Leu + Isoleu:400	Novel
P21	M	c.[633 + 1 G > A] + [633 + 1 G > A]	_	BCKDHB	3 D	Yes	Poor feeding, irritability, seizure	MSUD pattern	Val:389 Leu + Isoleu/phe:108 Leu + Isoleu:3670	(Abiri et al. 2017)
P22	M	c.[477 + 1 G > A] + [477 + 1 G > A	_	BCKDHB	20 D	Yes	Poor feeding, Seizures	MSUD pattern	Leu: 371.4 Isoleu:182.9	(Hayashida et al. 1994)
P23	M	c.[357delT] + [357delT]	p.[Leu119 > Lfs] + [Leu119 > Lfs]	BCKDHB	6 D	Yes	Poor feeding, seizure, irritability, maple syrup urine odor	MSUD pattern	Leu + Isoleu/phe:89 Leu + Isoleu = 2654	Novel
P24	M	c.[143delT] + [143delT]	p.[Leu48Argfs-X14] + [Leu48Argfs-X14]	BCKDHA	2 M	Yes	Poor feeding, lethargy, seizure	MSUD pattern	Leu + soleu:2927	(Abiri et al. 2016)
P25	F	c.[731G > A] + [731G > A]	p.[Gly244Glu] + [Gly244Glu]	BCKDHA	4 D	Yes	Poor feeding, hypoglycemia, seizure	MSUD pattern	Val:311 Leu + Isoleu/phe:108 Leu + Isoleu:4003	(Abiri et al. 2016)
P26	M	c.[702delT] + [702delT]	p.[Tyr235Thrfs-X94] + [Tyr235-ThrfsX94]	BCKDHA	6 M	Yes	Poor feeding, irritability, seizure	MSUD pattern	Val:632 Leu + Isoleu/phe:66 Leu + Isoleu:825	(Abiri et al. 2016)
P27	M	c.[1167 + 1G > T] + [1167 + 1G > T]	_	BCKDHA	10 D	Yes	Poor feeding, maple syrup urine odor, lethargy, hyporeflex	MSUD pattern	Val:938 Leu + Isoleu/phe:9.1 Leu + Isoleu = 2866	(Abiri et al. 2016; Gorzelany et al. 2009)
P28	M	c.(375 + 1_376–1)_ (884 + 1_885–1)del	_	BCKDHA	4.5 M	Yes	Poor feeding, seizure, irritability, maple syrup urine odor	MSUD pattern	Val = 344 Leu + Isoleu/phe = 67 Leu + Isoleu = 3967	(Abiri et al. 2016)
P29	M	c.[288 + 1G > A] + [288 + 1G > A]	_	BCKDHA	2 M	Yes	Poor feeding, seizure, hypertonia, irritability	MSUD pattern	Val = 473 Leu + Isoleu/phe = 143 Leu + Isoleu = 3494	(Abiri et al. 2016)
P30	M	c.[355-356 Ins 7 nt] + [355–356 Ins 7 nt]	p.[D355D fs] + [D355D fs]	BCKDHA	5 D	Yes	Poor feeding, irritability, seizure	MSUD pattern	Val:601.25 Leu + Isoleu/phe:108 Leu + Isoleu:4003	Novel
P31	M	c.[452C > T] + [452C > T]	p.[Thr151Met] + [Thr151Met]	BCKDHA	1 M	Yes	Seizures, poor feeding, maple syrup urine odor	MSUD pattern	Val:467.8 Leu + Isoleu/phe:108 Leu + Isoleu:4003	(Henneke et al. 2003)
P32	M		_	BCKDHA	42 D	Yes	Poor feeding, hypotonia	MSUD pattern	Val:278	(Abiri et al. 2016)

Table 1 (continued)

Patient ID	Sex	Genotype	Amino acid change	Gene	Age at diagnosis	Consanguinity Of parents	Clinical symptoms	GC-MS organic acids*	BCAA at diagnosis (μmol/L)**	References
		c.[288 + 1 G > A] + [288 + 1 G > A]							Leu + Isoleu/phe:94 Leu + Isoleu = 3906	
P33	M	c.[890G > A] + [890G > A]	p.[Arg297His] + [Arg297His]	BCKDHA	16 M	Yes	Poor feeding, seizure, irritability, maple syrup urine odor	MSUD pattern	Leu:1927.4	(A et al. 2000)
P34	M	c.[703delT] + [703delT]	_	BCKDHA	4 D	Yes	Poor feeding, seizure, irritability, maple syrup urine odor	MSUD pattern	Val:125.3 Isoleu:65 Leu:337.7	Novel
P35	M	c.[562G > T] + [562G > T]	p.[Gly188Trp] + [Gly188Trp]	BCKDHA	4.5 M	Yes	Poor feeding, seizure, irritability, maple syrup urine odor	MSUD pattern	Val:423 Leu + Isoleu/phe:108 Leu + Isoleu:4003	(Yang et al. 2012)
P36	M	c.[1291C > T] + [1291C > T]	p.[Arg431X] + [Arg431X]	DBT	4 D	Yes	Poor feeding, Seizures	MSUD pattern	Val:670 Leu + Isoleu/phe:108 Leu + Isoleu:4003	EGL genetics
P37	M	c.[363delCT] + c.[1238 T > C]	p.[Leu121Leu fs] + [Ile413Thr]	DBT	9 M	Yes	Sever DD, seizures, poor feeding, lethargy, maple syrup urine odor	MSUD pattern	Val:259.57 Isoleu:129.07 Leu:235.58	Novel
P38	M	c.(433 + 1_ 434–1)_ (939 + 1_ 940–1)del	_	DBT	14 D	Yes	Poor feeding, hypotonia	MSUD pattern	Val:215 Isoleu:158.8 Leu:319.5	Novel
P39	M	c.[1174A > C] + [1174A > C]	p.[Thr392Pro] + [Thr392Pro]	DBT	15 D	Yes	Poor feeding, irritability, seizure	MSUD pattern	Val:534.8 Leu + Isoleu/phe:108 Leu + Isoleu:4003	Novel
P40	M	c.[85_86ins AACG] + [85_86ins AACG]	–	DBT	3D	Yes	Poor feeding, irritability, seizure, bradypnea	MSUD pattern	Leu: 386 Isoleu:164.5	Novel

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-Methylvalerate, 2-ketoisocaproate

**Leucine +Isoleucine cut off <350 μmol/L, Leu + Isoleu/phe cut off <10 μmol/L, Val cut off <250 μmol/L.

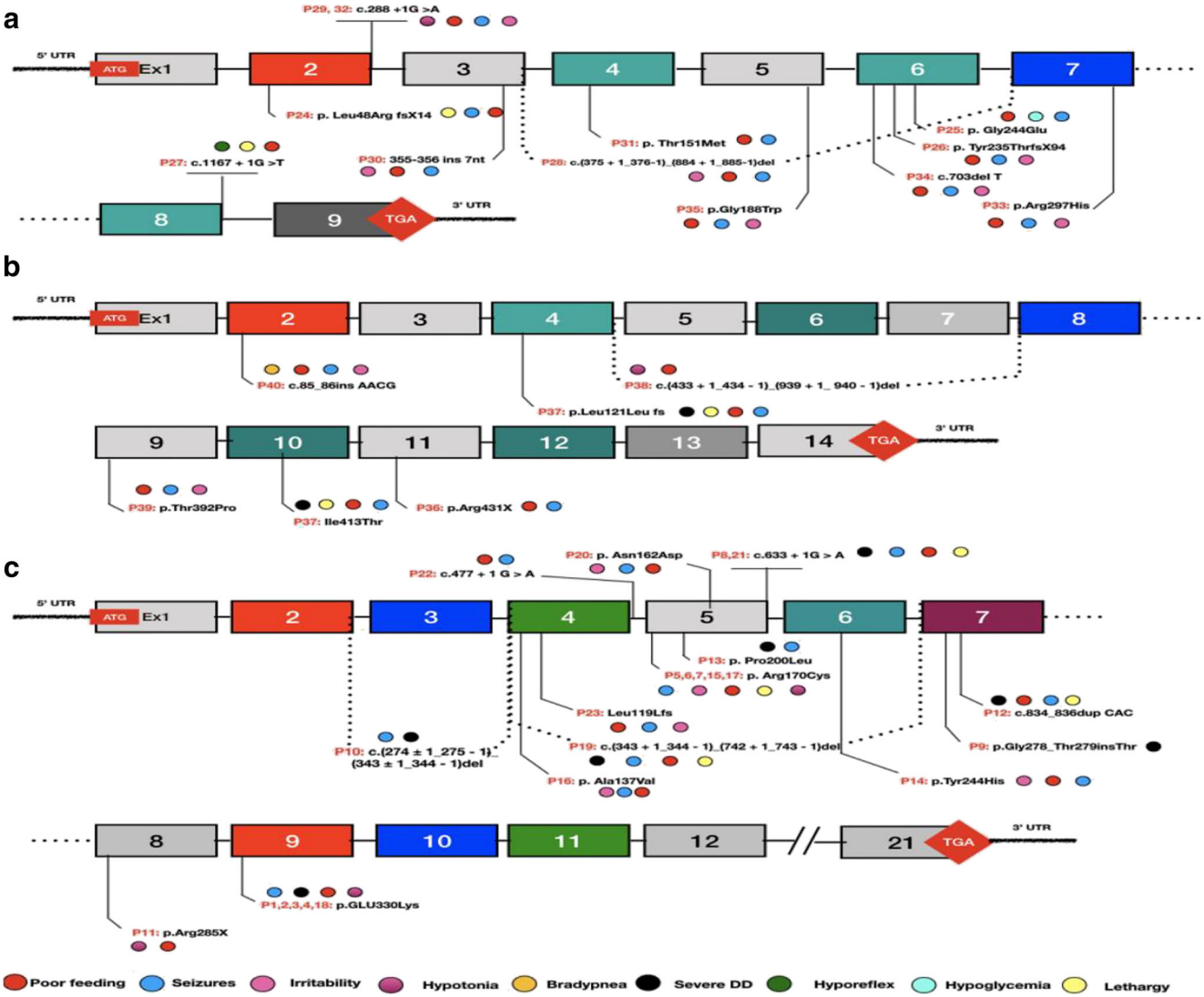

Fig. 1 Organization of the *BCKDHA (A)*, *BCKDHB (C)*, and *DBT (B)* cDNA consists of 9, 21, and 14 exons respectively, with positions of the mutations identified in this study. The numbers in red is the patients number in the Table 1. Colored circles near each identified mutation is demonstrate the severity of the disease based on the manifested phenotype

(Kawsar Biotech Co., KBC, Tehran, Iran, KBC), 0.3 each primer (10 M), 0.4 dNTPs (40 mM), 0.66 $MgCl_2$ (100 mM), 50–100 ng DNA and adequate ddH2O were added to prepared 25 ul final reaction mixture. The optimal PCR conditions included an initial denaturation step for 5 min at 95 °C, 1 min at 95 °C, 1 min at 62 °C,1 min in 72 °C and final extension for 10 min at 72 °C for 30 cycles. Sequencing was done according to the manufacturer's protocol of BigDye Terminator kit (Thermo Fisher Scientific, Life Technologies, USA, TS) and samples were run on ABI 3130 XL Genetic Analyzer by Kawsar Biotech Company. Chromas v2.33 software (Technelysium, Tewantin, Qld, Australia) was used for data visualization and data analysis performed by gene runner v6.0.

Mutation analysis

Sequences were aligned with the reference human genomic and cDNA sequences of MSUD genes (with the accession number; *BCKDHB* gene(NM_183050.2), *BCKDHA* (NM 000709.3), *DBT* (NM 001918.3), *DLD* (NM 000108.4). HGVS, Human Genome Variation Society, was used to the nomenclature of novel mutations (den Dunnen and Antonarakis 2000).

To identify the pathogenicity of the missense variants, various prediction tools (in silico) were used i.e. polyphen-2 (http://genetics.bwh.harvard.edu/pph2) (Adzhubei et al. 2010), SIFT (http://sift.jcvi.org) (Kumar et al. 2009), and Mutation tester (http://www.mutationtaster.org) which indicates that these mutations are damaging. To predict the impact of splice variant on splicing, Human splice Finder (http://www.umd.be/

HSF3/HSF.shtml) were used. Segregation analysis was performed to proof the pathogenicity of the identified mutations.

Computational analysis

Primary conformation for structural analysis was obtained from RCSB (PDBID: 2J9F). Gapped residues within the structure were repaired using Modeller 9.13 (Eswar et al. 2007). Hydrogen bonds were added by reduce 3.23 (Word et al. 1999). Mutations were induced by psfgen plugin of Visual Molecular Graphics (VMD) 1.9.3 (Humphrey et al. 1996); then all structures were minimized to remove bad contact using conjugate gradient algorithm for 1000 steps by NAMD 2.12 (Phillips et al. 2005). Final snapshots used for further analysis.

All analyses were implemented by the R statistical language. VMD 1.9.3 was used for structural representation. Hydrogen bonds and salt bridges were measured with H-Bonds and Salt Bridges plugins of VMD 1.9.3, respectively.

Results

In current study we aimed to summarize our previous findings and present ten novel mutations in forty families with clinical diagnosis of the classic form of the MSUD disease base on their clinical manifestations which is the most severe form. Twenty-three patients showed homozygous haplotypes for markers flanking the *BCKDHB* gene, twelve for *BCKDHA*, five for *DBT* and no family was homozygous for the markers of the *DLD* gene.

In investigated families most of the disease-causing mutations were restricted to exons and exon/intron boundaries of the BCKDHA, BCKDHB, and DBT gene, which allowed successful identification of the disease etiology. Table 1 Shows both the mutation and the related clinical data for each patient.

Analysis of twenty-three families with the homozygote haplotype of the *BCKDHB* gene revealed four families with the same haplotype structure, and further analysis showed the same mutation (c.988G > A). Three of the other families showed another haplotype with the same mutation of (c.508G > T). Rest of the families showed a different haplotype and also a different mutation including: Eight point mutation; (c.484 A > G), two (c.508 C > T), (c.599 C > T), (c.853 C > T), (c.988 G > A), (c.730 T > C), and (c.410 C > T). Two insertion; (c.833-834ins CAC), and (c.834_836dup CAC). Three splice site mutation; two (c.633 + 1 G > A), and one (c.477 + 1 G > A). A single nucleotide deletion, (c.357delT), a homozygote deletion of exon 3, c. (274 + 1_275–1) _ (343 + 1_344–1)del, and a homozygote deletion of exons 4,5,6, c. (343 + 1_344–1) _ (742 + 1_743–1)del.

As mentioned, *twelve* families were linked to the *BCKDHA* gene. Sequence analysis of the *BCKDHA* gene revealed twelve different mutations. From these, three were deletion (c.143delT), (c.702delT), and (c.703delT). Four were point mutation (c.731 G > A), (c.890 G > A), (c.452 C > T), and (c.562 G > T). Three splice site mutations, a (c.1167 + 1G > T), and two (c.288 + 1 G > A). An insertion (c.355–356 ins 7 nt ACAAGGA), and one homozygote deletion of exon 4 of the gene c. (375 + 1_376–1) _ (884 + 1_885–1)del.

As indicated before, five families showed homozygous haplotype for markers flanking the *DBT* gene. From these, two were point mutations; (c.1291 C > T) and (c.1174 A > C). one compound heterozygote mutation including deletion and a point mutation c.[363del CT; 1238 T > C]. one insertion(c.85_86ins AACG (, and homozygote deletion of exon 5,6,7 of the gene c. (433 + 1_ 434–1) _ (939 + 1_ 940–1)del.

Interestingly, we found ten novel mutations that have not been reported previously (Table 2). Among the novel

Table 2 Mutations identified in this study

Gene	Mutation	Protein	SIFT Prediction (Cutoff = 0.05)	Polyphen	Mutation Tester	ACMG classification
BCKDHB	c.484 A > G	p.Asn162Asp	Damaging	Damaging	Disease causing	Pathogenic (PS)
BCKDHB	c.834_836dup CAC	_	Damaging	Damaging	Disease causing	Pathogenic (PS)
BCKDHB	c.(343 + 1_ 344–1)_(742 + 1_ 743–1)del	_	_	_	Disease causing	Pathogenic (PVS1)
BCKDHB	c.357delT	p.F120Lfs*110	Damaging	Damaging	Disease causing	Pathogenic (PVS1)
BCKDHA	c.355_356 ins 7 nt ACAAGGA	p.D335D fs	_	_	Disease causing	Pathogenic (PVS1)
BCKDHA	c.703delT	p.Y235Tfs*95	Damaging	Damaging	Disease causing	Pathogenic (PVS1)
DBT	c.1238 T > C	p.I413Thr	Damaging	Damaging	Disease causing	Pathogenic (PS)
DBT	c.(433 + 1_ 434–1)_(939 + 1_ 940–1)del	_	_	_	Disease causing	Pathogenic (PVS1)
DBT	c.85_86ins AACG	p.V29Efs*21	_	_	Disease causing	Pathogenic (PVS1)
DBT	c.1174 A > C	p.Thr392Pro	Damaging	Damaging	Disease causing	Pathogenic (PS)

mutations, five of them were in *BCKDHB* gene including one point mutations (c.484 A > G), a deletion (c.357del T), an insertion (c.834_836dup CAC), and homozygote deletion of exons 4,5,6 of the gene. In the *BCKDHA* gene, we also found two novel mutations including, (c.703del T), and (c.355–356 ins 7 nt ACAAGGA). Four novel mutations that were identified in the *DBT* gene are)c.1238 T > C), homozygote deletion of exon 5,6,7, c. (433 + 1_ 434–1) _ (939 + 1_ 940–1)del, (c.1174 A > C), and (c.85_86ins AACG). The rest of the mutations were reported previously.

Geographic distribution of families in Iran with BCKD mutations are shown in Fig. 2. We divided a geographic map of Iran into eight different regions as follows; northwest, north, northeast, central, west, southwest, southeast, and south. MSUD were identified in patients from northwest, west, north, central and southwest, most frequently in the northwestern province **(**Fig. 2**).** The distribution of mutations is shown in Figs. 1 and 3**.**

We checks each identified mutations on fifty Iranian unaffected individuals from the same ethnic background, to determine the pathogenicity of novel variant alleles. We also assessed the impact of novel mutations as well as large deletions on protein structure and function by computational analysis.

Overall 3D structure of complex and represented mutation was shown in Fig. 4. N162D reduced BB' (*BCKDHB*) inter-subunit hydrogen bond interactions due to the elimination of ASN162 (B′)—ASP151(B) and TYR195(B)—ASN162(B′) residues **(Table S1).** Instead, intra-subunit salt-bridge interactions within the B subunit was formed **(Table S2).** P200L mutation might contribute to the flexibility of adjacent helix.

Discussion

Approximately 40% of the amino acids required by mammals are branched-chain amino acids (BCAAs) such as valine, leucine, and isoleucine. BCAAs can be used as a source of energy through an oxidative pathway that uses a α-ketoacid as an intermediate. Decarboxylation of α-ketoacids is mediated by a multimeric enzyme complex called "branched-chain α-ketoacid dehydrogenase (BCKD)". The BCKD complex is composed of at least four catalytic components and two regulatory enzymes, which are encoded by six genes. A

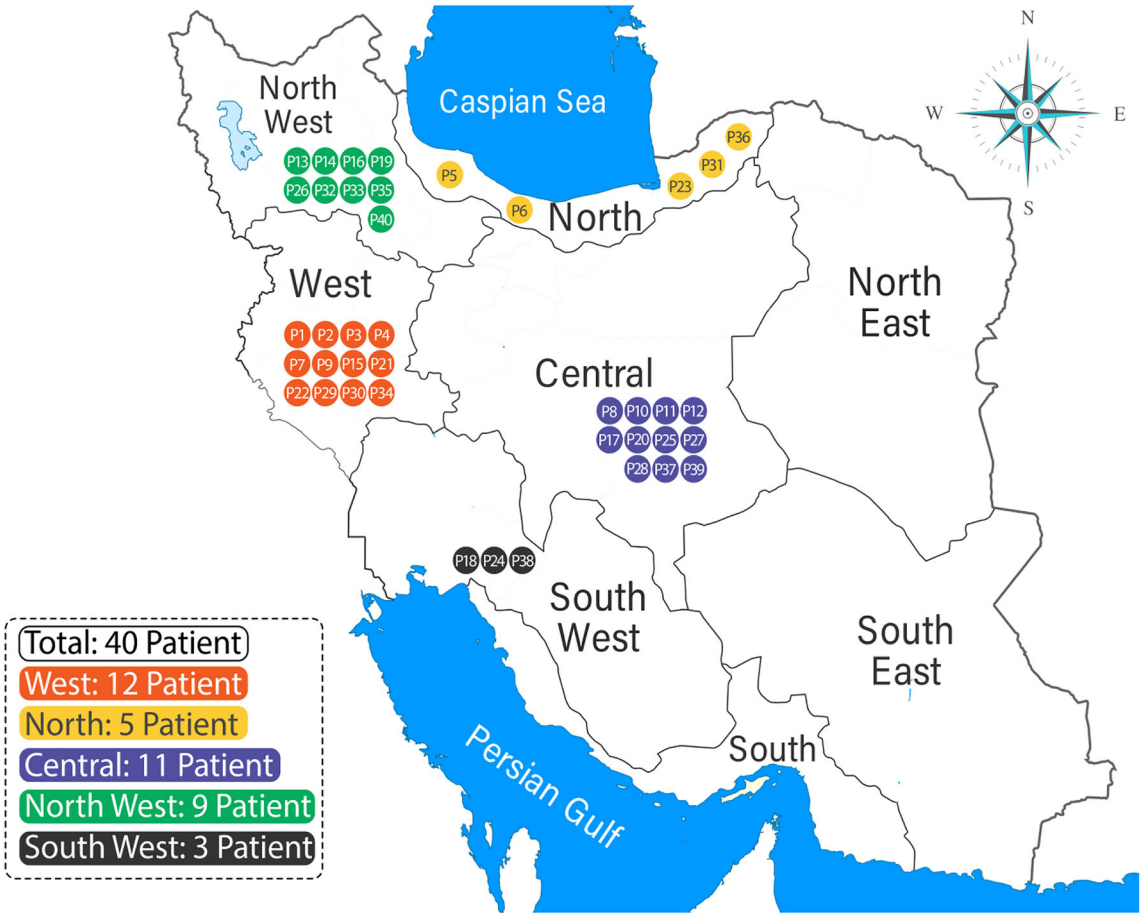

Fig. 2 Geographic distribution of families in Iran with BCKD mutations are shown. We divided geographic map of Iran into eight different regions. MSUD were identified in patients from north west, west, north, central and south west, most frequently in the northwestern province

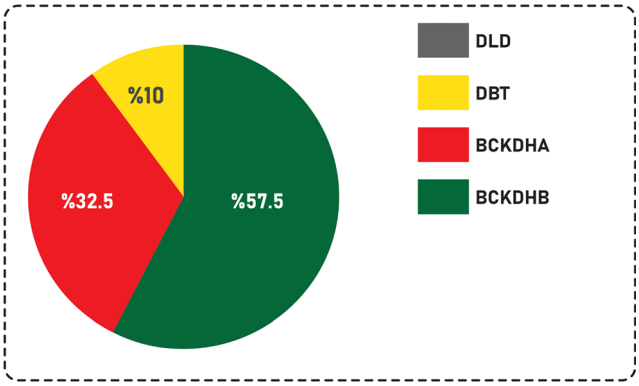

Fig. 3 The pie chart, which is divided into three slices used to illustrate the numerical proportion of the mutations occurred in each distinct gene (BCKDHA, BCKDHB, DBT and DLD) responsible for MSUD in the studied population

deficiency of any one of these six components leads to a disorder known as maple syrup urine disease (MSUD), so named because the urine of affected person has an odor reminiscent of maple syrup **(**Fig. 5**).**

Treatment of MSUD consists of dietary restriction of BCAAs to the minimum required for normal growth. Untreated patients with MSUD accumulate BCAAs and their associate ketoacids, leading to progressive neurodegeneration and death in the first few months of life. There are two other therapeutic strategies for MSUD. First, removing the toxic substance from the body and symptomatic treatment, especially in the acute phase, including peritoneal dialysis, hemodiafiltration or hemodialysis, a high rate glucose infusion to stimulate insulin secretion and suppress protein catabolism. Levocarnitine could increase the plasma free carnitine and decrease the malondialdehyde to protect the neuron (Ribas et al. 2014). Second, is that increases BCKD activity via tissue or cell transplantation, such as liver transplantation (Al-Shamsi et al. 2016; Miryounesi et al. 2015).

The prevalence of MSUD in the general population is low, but MSUD is relatively common in consanguineous populations. MSUD is relatively common in the Mennonite community of Lancaster Country, Pennsylvania, where approximately 1 in 7 persons is a heterozygous carrier (Strauss et al. 2012). This is an example of the founder effect in a small, relatively isolated population. The rate of consanguineous marriage in Iran is about 38% (Saadat et al. 2004), therefore, the rate of autosomal recessive disorders will be higher in comparison to western countries (Teeuw et al. 2014). MSUD is a rather rare disease in Iran with a high rate of consanguinity. In this regard, very few studies have been published about mutation spectrum in Iran (Miryounesi et al. 2015; Zeynalzadeh et al. 2018), considering this, we conducted homozygosity mapping for forty families with MSUD.

Different molecular techniques can be applied for diagnosis of patients and carriers, such as Sanger sequencing (Zeynalzadeh et al. 2018), real-time qPCR, STR linkage analysis (Abiri et al. 2017), and high-throughput sequencing (Li et al. 2018). Homozygosity mapping with the help of STRs is an efficient tool for quick determination of a single gene involved in genetically heterogeneous disorders such as MSUD.

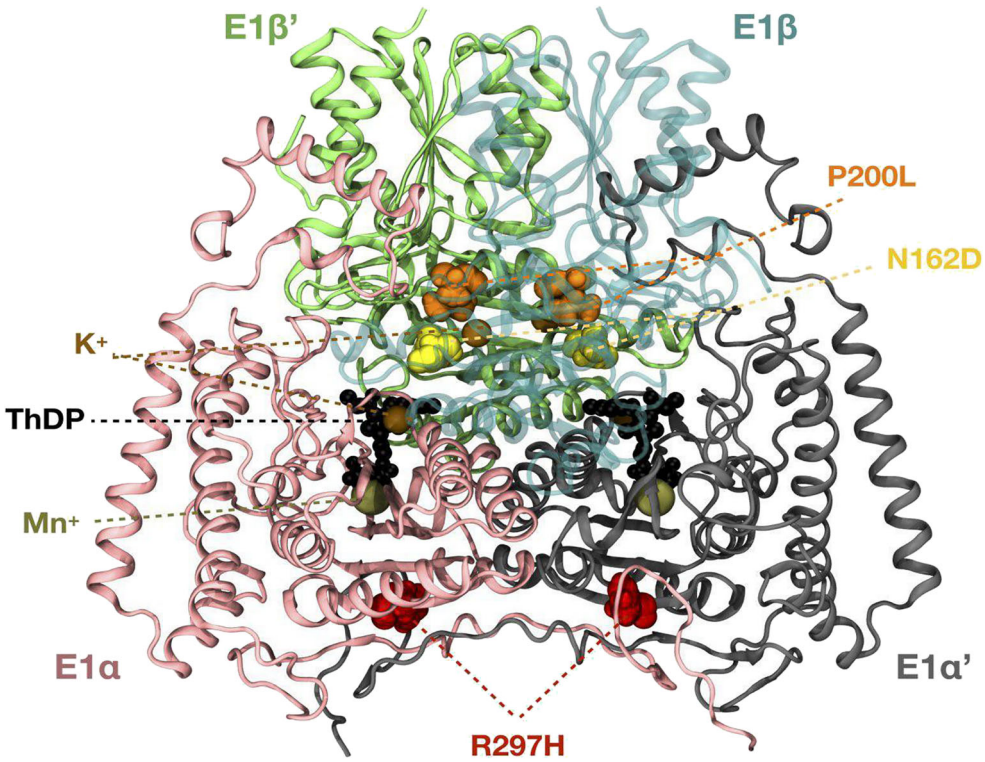

Fig. 4 A) Structural representation of E1b heterotetramer: E1α (pink); E1β (cyan); E1α′ (grey) and E1β′ (green). The mutated residues – N162D-β/β', P200L-β/β'and R297H-α/α'are indicated in yellow, orange and red; ThDP, Magnesium and potassium ions are presented in black, tan and brown

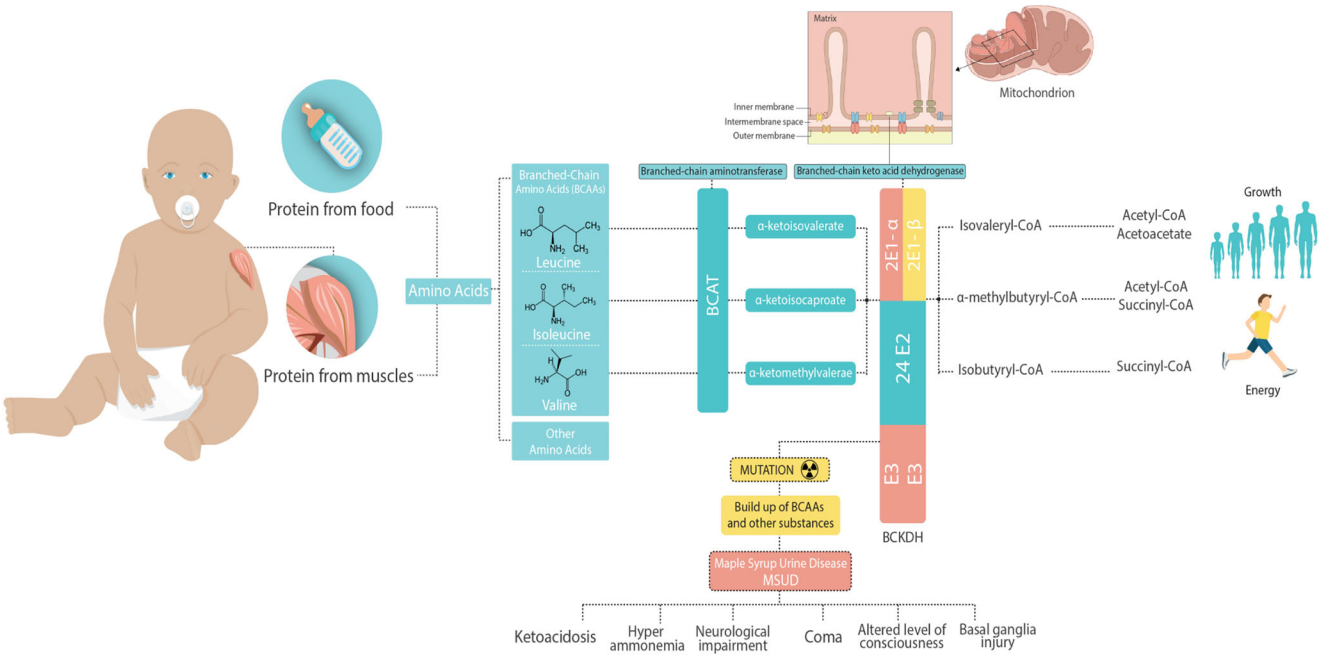

Fig. 5 Schematic representation of the pathology of maple syrup urine disease, depicting the catabolic pathway of branched-chain aminoacids; Valine, leucine, and Isoleucine (BCAAs). BCAAs metabolism depends on the proper activity of Branched-chain keto acid dehydrogenase complex, a mitochondrial enzyme, which consists of four catalytic subunits (E1α, E1β, E2, and E3). Deficiency in any one of the mentioned subunits due to a genetic mutation, results in accumulation of the BCAAs and their 2-oxoacids in the body fluid. Thus, if the disease remains untreated, the following ketoacidosis, hyper ammonemia, and Basal ganglia injury may lead to the cognitive deficits and movement disorders, which are permanent in those who have had episodes of severe metabolic decompensation

This tool is especially useful in a population with a high rate of consanguineous marriages.

Haplotype analysis of the studied families showed twenty-three families with homozygous haplotype block for the BCKDHB gene, twelve for BCKDHA, five for DBT, and no family was homozygous for markers flanking the DLD gene. Unlike the general population, that the mutations responsible for MSUD disease are in BCKDHA gene, the most frequent mutations in the MSUD patients from a consanguineous population of Iran was shown in BCKDHB gene (Fig. 3). Subsequently, we sequenced the corresponding gene in each family. Ten novel mutations were identified in these responsible genes as follows.

Two missense mutations were identified in the BCKDHB gene which encodes for E1b subunit. First, was p. P200L mutation which lead to the substitution of proline with leucine, might contribute to less flexibility with adjacent helix (B′) (Fig. 4). The second missense mutation was p. N162D which lead to substitution of asparagine with aspartic acid, leads to reduced BB' (BCKDHB) inter-subunit hydrogen bond interactions due to the elimination of ASN162 (B′)—ASP151(B) and TYR195(B)—ASN162(B′) residues (Fig. 4). Instead, intra-subunit salt-bridge interactions within the B subunit was formed (**Table S1 and S2**).

Another mutation identified in this gene was (c.357del T), that leads to a frame-shift mutation and premature stop codon (position of a stop codon in WT/Mut CDS 1179/687) and protein loss of function. We also identified a large homozygote deletion of exon 4,5, and 6 of the gene, which leads to Thiamin diphosphate-binding fold (THDP-binding) and transketolase-like, pyrimidine-binding domain elimination, which is critical for proper activity of the protein. A novel insertion (c.834_836dup CAC) were identified in this gene that inserts a histidine amino acid at the 279 aa position of the protein. Human splice finder predicted that the insertion will alter the wild-type donor splice site and probably affect splicing (Desmet et al. 2009). HSF prediction algorithms that it increases the consensus value of wild type from 15.8 to 68.39 for the mutant. Also, the predicted variation score is +332.85% (this prediction is much higher than +10% in HSF).

In BCKDHA gene we identified a seven nucleotide insertion mutation (p.D335D fs), which leads to a frame-shift mutation and premature stop codon (position of a stop codon in WT/Mut CDS 1338/369) resulting in a truncated protein of only 123 residues and the protein loss of function (Hu and Ng 2012). This is considerably shorter than the wild type with 446 residues. Since the stop codon happens in an aberrant position, the resulting mRNA will be degraded by nonsense-mediated-decay (NMD). Therefore, the usual result of nonsense mutation is to prevent any expression of the gene (Wynn et al. 1998). The second mutation identified in this gene was (c.703del T), which cause a frame-shift mutation as well as

premature stop codon leads to a truncated protein formation with 329 residues out of 446.

Four families showed distinct mutations at DBT gene. The first family showed a missense mutation, (c.1174 A > C) in which, the substitution of threonine with proline. The mutated residue is located in 2-Oxoacid dehydrogenase acyltransferase catalytic domain and in contact with E2 domain. The mutation can affect this interaction and as such effect protein function. Second mutation was (c.1238 T > C), that leads to the substitution of isoleucine with threonine in the conserved domain of the protein and my effect protein structure, for the mutant amino acid is smaller in size and is less hydrophobic than wild type aa so it will cause loss of hydrophobic interactions in the core of the protein. The third mutation was an insertion (c.85_86ins AACG) that leads to a frame-shift mutation and premature stop codon (position of a stop codon in WT/Mut CDS 1449/147) and the resulting mRNA will be degraded by nonsense-mediated-decay (NMD). The last family showed a homozygote deletion of exons 5, 6, and 7 of the gene. Despite, the gross deletion effects catalytic domain of the protein, remaining a single nucleotide at the end of exon 4 for codon 147 leads to a frame-shift mutation, that change the amino acid sequence from the aa 314 to aa 482.

None of the identified variants are present in the single nucleotide polymorphism database (Exome Variant Server, http://evs.gs.washington.edu/EVS/), Iranome (http://www.iranome.ir), Exome Aggregation Consortium ExAC (http://exac.broadinstitute.org) and 1000 genome project (1000genomes.org).

The missense mutation c.853 C > T identified in one of our patients and previously described as pathogenic by Henneke et al. (2003). Since it leads to the premature termination codon. C.452C > T mutation was also identified by Henneke et al. and reported to be pathogenic and responsible for a classic form of the disease. The mutation c.730 T > C that identified in one of our patients was previously reported by Flaschker et al. (2007), and described as pathogenic since it affects the cofactor binding site of the protein. Mutation c.410 C > T is found in our patient and previously reported by Nellis et al. (2003), to be pathogenic because of decreasing subunit interactions and the inability to form tetrameric interactions. Another mutation c.508 C > T that was reported by Jung Min et al. (2014), identified in one of our patients and reported to be pathogenic so because of its effect on the highly conserved pyrimidine-binding domain. C. 988 G > A mutation was previously identified by Safdarian et al. (2016), and reported to be pathogenic based on the conformation changed that corresponded by mutation might effects complex building between BCKDH and its partners.

The splice site mutation c.477 + 1 G > A was identified in one of our patients and previously reported by Hayashida et al. (1994). This mutation leads to the deficiency of E1 beta subunit due to a single base substitution of the intron 5, resulting in two alternatively spliced mRNA in a patient. The missense mutation c.562 G > T was identified in a patient and was previously reported to be probably damaging by Yang et al. (2012), so due to the mutation in highly conserved subunit interface domain.

In recent years, some cases with MSUD have been found in the Iranian population by the promotion of homozygosity mapping (Abiri et al. 2016, 2017), and sequencing (Miryounesi et al. 2015). Some molecular data was obtained showing that mutations in the BCKDHB gene were most common in Iranian patients with MSUD (Fig. 3), which were different from other reports in other populations (Morton et al, 2002). In this survey, we summarized our previous reports about MSUD in Iranian population (Abiri et al. 2016, 2017), and the variants happened in BCKDHA, BCKDHB, and DBT genes as well as in the silico analysis of novel mutations that have been never reported before. However, gross deletion, insertion, and point mutations are the common mutation in the present study, that equally distribute between three responsible genes, but the cases with BCKDHB mutations were in the majority in our studied population (Fig. 3).

Conclusion

All in all, we present the clinical characteristics and mutation analysis of 40 patients with MSUD, which had been identified by homozygosity mapping and sequencing. Ten novel mutations were found in BCKDHB, BCKDHA, and DBT genes and in silico analysis was conducted for predicting the pathogenicity of these mutations. Identification of these mutations in this study further expands the spectrum of known gene mutation and contributes to the genotype-phenotype correlation and prenatal molecular diagnosis of MSUD.

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Compliance with ethical standards

Conflict of interest The authors declared no potential conflict of interests.

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