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Genetic analysis by targeted next-generation sequencing and novel variation identification of maple syrup urine disease

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Genetic analysis by targeted next-generation sequencing and novel variation identification of maple syrup urine disease

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Abstract

Maple syrup urine disease (MSUD) is a rare autosomal recessive disorder that affects the degradation of branched chain amino acids (BCAAs). Only a few cases of MSUD have been documented in Mainland China. In this report, 8 patients (4 girls and 4 boys) with MSUD from 8 unrelated Chinese families were diagnosed at the age of 9 days to 1 year and 8 months. All the coding regions and exon/intron boundaries of *BCKDHA*, *BCDKHB*, *DBT*, *DLD* genes were analyzed by targeted NGS in the 8 MSUD pedigrees. Targeted NGS revealed 2 pedigrees with MSUD Ia, 5 pedigrees with Ib, 1 pedigree with MSUD II. Totally, 13 variants were detected, including 2 variants (p.Ala216Val and p.Gly281Arg) in *BCKDHA* gene， 10 variants (p.Gly95Ala, p.Ser171Pro, p.Phe175Leu, p.Arg183Trp, p.Lys222Thr, p.Arg285Ter，p.Arg111Ter，p.S184Pfs*46, p.Arg170Cys, p.I160Ffs*25) in *BCKDHB* gene, 1 variants (p.Arg431Ter) in *DBT* gene. In addition, 4 previously unidentified variants (p.Gly281Arg in *BCKDHA* gene, p.Ser171Pro, p.Gly95Ala and p.Lys222Thr in *BCKDHB* gene) were found. NGS plus Sanger sequencing detection is effective and accurate for making gene diagnosis. Computational structural modeling indicated that these novel variations might affect structural stability.

Introduction

Maple syrup urine disease (MSUD, OMIM # 248600) is a hereditary branched-chain amino acid metabolism disorder caused by branched chain α-ketoacid dehydrogenase multi-enzyme complex (BCKDC). Common clinical manifestations of MSUD are feeding difficulties, epilepsy, mental retardation, ketonuria and maple-like body odor. Without timely intervention, the disease progresses rapidly, and the mortality and disability rate are very high. According to the phenotype, MSUD can be divided into 5 type[1]: classic, intermediate, intermittent, thiamine-reactive and dihydrolipoylamide dehydrogenase (E3) deficiency type. The classic type is the most common and

severe type in the neonatal period, accounting for 75% affected infants. It usually occurs 4-9 days after birth. The liver BCKDC activity in classic type children is often lower than 2% in healthy children, manifested as ketoacidosis, neurologic damage and mental retardation[2]. 20% are intermediate or intermittent type, the intermediate type usually shows a continuously increased concentration of branched-chain amino acids (BCAAs), accompanied by nervous system damage[3]. Intermittent type usually occurs from 5 months to 2 years with mild symptoms[4], prognosis of the thiamine-responsive type is better than classic type, with BCKDA activity of 2% -40%, and some children can survive for a long time[5]. The activity of dihydrolipoamide acyl dehydrogenase (E3) -deficient BCKDA is 25% lower than that of normal children, and it is characterized by low tension, stunting, and lactic acidosis[6].

MSUD is inherited in autosomal recessive pattern, and it is very rare in most populations, with an incidence of 1:185000[7]. BCKDC is located in the mitochondrial inner membrane and consists of 4 subunits Elα, E1β, E2, E3, which are encoded by *BCKDHA, BCKDHB, DBT* and *DLD* genes, respectively[8]. According to the involved subunit, MSUD is divided into the following types: (1) type Ia (OMIM 608348), caused by biallelic pathogenic variants in *BCKDHA* gene encoding the Elα subunit; (2) type I b (OMIM 248611), caused by biallelic pathogenic variants in *BCKDHB* gene encoding the E1β subunit; (3) type II (OMlM 248610), caused by biallelic pathogenic variants in *DBT* gene encoding E2 subunit; (4) type III (OMIM 238331), caused by biallelic pathogenic variants in *DLD* gene encoding E3 subunit[7]. Another two subtypes Type IV and type V are specific kinase and phosphatase gene mutation types, respectively.

MSUD is a genetically heterogeneous disease, and the traditional sequencing technology is time-consuming and costly. High-throughput sequencing technology based on target gene capture for sequencing of the four genes can simultaneously detect gene mutations in the causative genes, not only providing accurate genetic diagnosis results for patients, but also providing clinicians with the basis for differential diagnosis, drug treatment and subsequent genetic counseling, and prenatal diagnosis. In this study, we applied targeted high-throughput sequencing to sequence the target regions of *BCKDHA, BCKDHB, DBT* and *DLD* genes in peripheral blood samples of patients or parents in 8 families with MSUD, and Sanger sequencing validation was subsequently performed for confirmation of suspected pathogenic variants.

Methods

Subjects. Eight unrelated families that had given birth to children affected with MSUD were collected. All children in the 8 families were screened by tandem mass spectrometry and received positive screening results. Only children in family 3 and 7 accepted timely diagnosis and treatment after neonatal screening. As is shown in Table 1, The remaining 6 children developed the disease from 2 days to 2 months, and died at 16 days, 2 months, 20 days, 1 month, 10 days and 1 month, respectively. Written informed consent was obtained from the legal guardians. All of the procedures and informed consent were approved by the Medical Ethics Committee of the First Affiliated Hospital of Zhengzhou University, and was performed according to the principles of the Declaration of Helsinki.

Table 1 Clinical and laboratory features of the 8 patients with MUSD

Patients	1	2	3	4	5	6	7	8
Sex	F	F	M	F	M	M	F	M
Age of Onset	7d	1m	4m	10d	2d	3d	17d	5d
Clinical manifestation								
Vomiting	+	-	-	+	-	-	-	-
Poor response	+	-	-	-	+	+	+	+
Coma	+	-	-	+	-	-	-	-
Convulsion	+	+	-	-	-	+	+	+
Feed difficulties	-	+	-	+	+	+	+	+
Hypermyotonia	-	+	-	-	-	+	+	-
Maple syrup odor	-	+	-	-	-	+	-	-
Mental retardation	-	-	+	-	-	-	-	-
Motor development delay	-	-	+	-	-	-	-	-
Seizure	-	-	-	-	+	-	-	-
Cranial CT/MRI	/	Extensive Low-density changes in the brain	Bilateral Frontotemporal parietal white mater density symmetry decreased	/	/	/	Poor myelination in white matter, diffuse long T1 and long T2 Edema signal changes in subcortical white matter and frontotemporal white matter	/
Blood tandem mass spectrometry								
Leucine/Isoleucin（μmol/L）	1904（<295）	935（<200）	1122.88（<375）	679（<230）	3089（<375）	3209（<200）	1754（<200）	2748（<300）
Valine	354（<270）	295（<210）	325.05（<325）	420（<230）	578（<325）	521（<280）	302.92（<280）	520（<300）
Leucine/Phenylalanine	34.2（<7）	19.4（<8）	29.92(<8)	12.6（<7）	38.2(<8)	39.1(<8)	22(<8)	29.5（<8）
Clinical phenotype outcome	Classic Died at 16d	ITD Died at 2m	ITD Mental retardation	ITD Died at 20d	Classic Died at 1m	Classic Died at 10d	ITD Mental retardation	Classic Died at 1m

Notes: F=Female, M=Male, d=day, m=month, ITD =Intermediate, ITM =Intermittent

Blood amino acid and ester Acylcarnitine Spectra analysis. Venous blood was collected from the children on an empty stomach for more than 4 hours. Blood amino acid and ester-acylcarnitine profiling was performed using liquid-tandem mass spectrometry.

DNA extraction. Blood samples (2 ml) were collected from each patient and their parents in families 3 and 7 by venipuncture in EDTA tubes. For the remaining six families, parental blood samples were collected. Genomic DNA was extracted from peripheral blood leukocytes using a QIAamp® DNA Blood Mini Kit (Qiagen, Cat.No.51106, Germany) according to the recommended protocol.

Next-generation sequencing. Using exon capture microarrays, metabolic disease gene panel was selected for high-throughput sequencing detection, with an average sequencing depth of 200 × and more than 97% of coverage at a depth of greater than 20 ×. Unique sequence reads were aligned with reference genome (GRCh37/hg19) to obtain variant files. The variants were filtered by comparing with NCBI dbSNP database, ExAC database and 1000 Genome database to exclude known polymorphisms with a frequency of more than 5% in normal subjects, and by searching the Human Gene Mutation Database (HGMD, http://www.hgmd.org/) to clarify whether the variant is a known pathogenic variant. The nomenclature of new variants was based on the international gene variant nomenclature system (http://www.hgvs.org/mutnomen).

Validation tests of Sanger sequencing. Gene tool software was used for designing primers for suspected variants. Routine PCR reactions were performed. PCR products were purified and directly sequenced on ABl3130-xl gene sequencing instrument using the ABIBigDye3.1 sequencing kit (Thermo Fisher Scientific, USA), and the sequencing data were compared and analyzed using ABI Sequencing Analysis 5.1.1 software.

***In silico* webservers and structure prediction.** Multiple sequence alignments were performed using HomoloGene database (http://www.ncbi.nlm.nih.gov/homologene) to verify the degree of conservation. The pathogenicity of the variants was then evaluated using three *in silico* webservers, PolyPhen2, SIFT and Mutation Taster2.The American College of Medical Genetics and Genomics(ACMG) guideline was applied to assess novel variants' pathogenicity. Computational modeling was carried out to observe the effect of new missense mutations on protein structure. Three-dimensional structure of the target protein sequence was constructed using PyMOL protein

model structure simulation software to determine the effect of amino acid substitution on protein structure.

Results

Molecular analysis in *BCKDHA*, *BCKDHB* and *DBT* genes. NGS was performed in children of families 3 and 7 and couples of the other 6 MSUD families to detect the sequence variation in each exon of the 4 causative genes (*BCKDHA, BCDKHB, DBT, DLD*) associated with MSUD. After alignment with the hg19 sequence, the variants were filtered by excluding the SNPs (normal frequency>0.05) reported in the dbSNP137 database, Hapmap database and 1000 Genome database. Suspected variants in the causative genes associated with the 8 families were shown in Table 2.

Sanger sequencing results. The suspected variants found by NGS were confirmed by Sanger sequencing. The patient in family 3 carried *BCKDHB* gene c.511T>C(p.Ser171Pro) and c. c.547C>T (p.Arg183Trp) compound heterozygous variants, and the children in pedigree 7 carried c.508C>T(p.Arg170Cys) and c.478-552del(p.I160Ffs*25) compound heterozygous variants. Their parents were heterozygous carriers of the respective variant. Heterozygous variants in the same causative gene of MSUD were detected in both couples in the remaining six families.Gene sequences of four novel variant in *BCKDHA* and *BCKDHB* genes were shown in Figure 1.

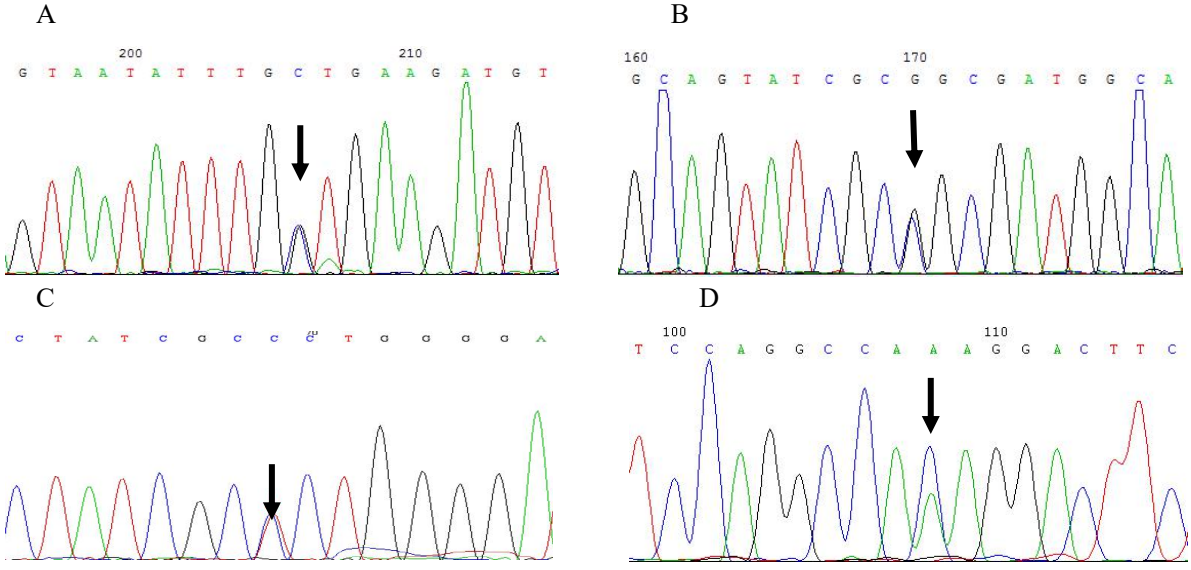

Figure 1: Gene sequences of four novel variant in *BCKDHA* and *BCKDHB* genes in eight pedigrees: c.841G>C(p.Gly281Arg) in *BCKDHA* gene(A), c.284G>C(p.Gly95Ala) in *BCKDHB* gene(B), c.511T>C(p.Ser171Pro) in *BCKDHB* gene(C), c.665A>C(p.Lys222Thr) in *BCKDHB*

gene (D)

***In silico* prediction of novel gene variant sequences.** Mutation Taster and PolyPhen-2 analysis showed that four novel missense variants:p.Gly95Ala, p.Gly281Arg, p.Lys222Thr and p.Ser171Pro were highly likely to be pathogenic/deleterious variants. We use The American College of Medical Genetics and Genomics(ACMG) guideline to assess these novel variants' pathogenicity in Table 2.

Table 2:Detection and analysis of gene mutation in 8 MSUD families

Pedigree	Gene	Proband genotype		Paternal genotype	Maternal genotype	Pathogenicity assessment based on ACMG guideline
		Genotype 1	Genotype 2			
1	*BCKDHA*	/	/	p.Gly281Arg#	p.Gly281Arg	PM3+PP3
2	*BCKDHA*	/	/	p.Ala216Val	p.Ala216Val	
3	*BCKDHB*	p.Ser171Pro	p.Arg183Trp	p.Arg183Trp	p.Ser171Pro#	PM3+PP3
4	*BCKDHB*	/	/	p.Phe175Leu	p.Lys222Thr#	PM3+PM5+PP3
5	*BCKDHB*	/	/	p.Gly95Ala#	p.Arg285Ter	PM3+PP3
6	*BCKDHB*	/	/	p.Arg111Ter	p.S184Pfs*46	
7	*BCKDHB*	p.Arg170Cys	p.I160Ffs*25	p.Arg170Cys	p.I160Ffs*25	
8	*DBT*	/	/	p.Arg431Ter	p.Arg431Ter	

Notes: #novel variation

Three-dimensional structure of proteins. The predicted three-dimensional structures of 4 novel variants in *BCKDHA* and *BCKDHB* genes were shown in Fig.2. In *BCKDHA* gene, Glycine 281 is located in the random coil structure of protein secondary structure. Glycine lacks side chain (only one H-bond). After mutation to arginine, arginine is a basic amino acid with ions, affecting the stability of E1α tertiary structure, thus affecting protein function.

In *BCKDHB* gene, As is shown in Fig.2F，Serine at position 171 is located in the α-helix of protein secondary structure, forming hydrogen bonds with Leucine at position 174, Phenylalanine at position 175 and Tyrosine at position 167. After mutation to Proline, the hydrogen bonds with Leucine at position 167 and Phenylalanine at position 175 disappear, affecting the stability of protein secondary structure. Therefore, it is speculated that p.Ser171Pro mutation has a greater impact on protein function.

Amino acid 222 is located in the α-helix of the secondary structure of the protein, amino acid 222 forms hydrogen bonds with amino acids 79, 83, 218, 225, 252, and 254.After mutation to Threonine, it reforms hydrogen bonds with amino acids 76, 218, and 225.The secondary structure of the protein is changed, which disrupts the stability of the protein and may affect the cleavage

and activation function of the protein.

Gly95 is located in the β-turn region, and due to the lack of side chains (only one H atom) by Glycine, there is no steric hindrance, allowing a U-shaped turn of the peptide chain by 180° in the β-turn. Mutation of Glycine to Alanine, which forms some steric hindrance, is likely to change the turning of the β-turn, causing change in local steric conformation.

A

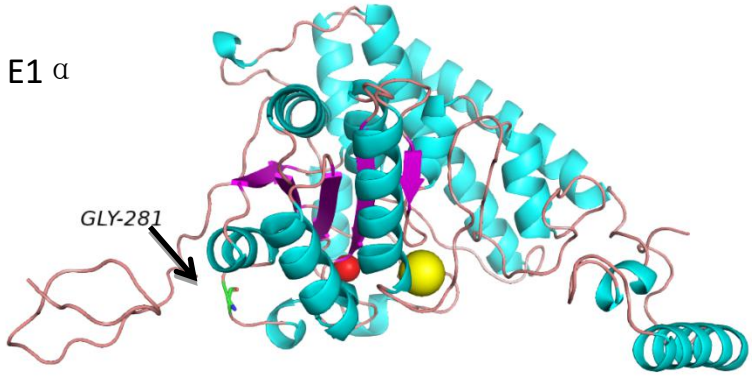

Fig.2.(A):Predicted 3D protein structure Of E1α compotent complex and the site of the Glycine(Gly281) colored in green

B

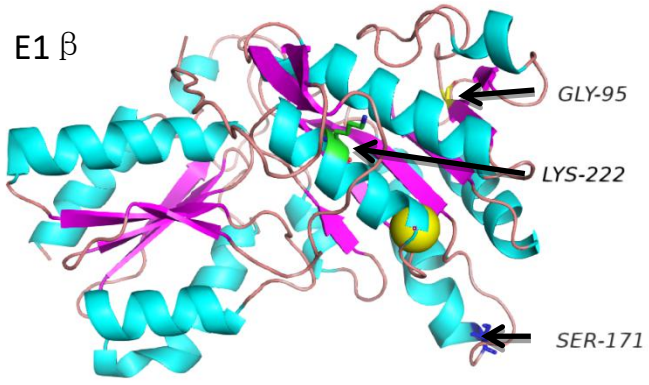

Fig.2.(B): Predicted 3D protein structure Of E1β compotent complex and the site of three residues.The Glycine(Gly95),Lysine(Lys222),Serine(Ser171) are colored yellow,green and blue respectively.

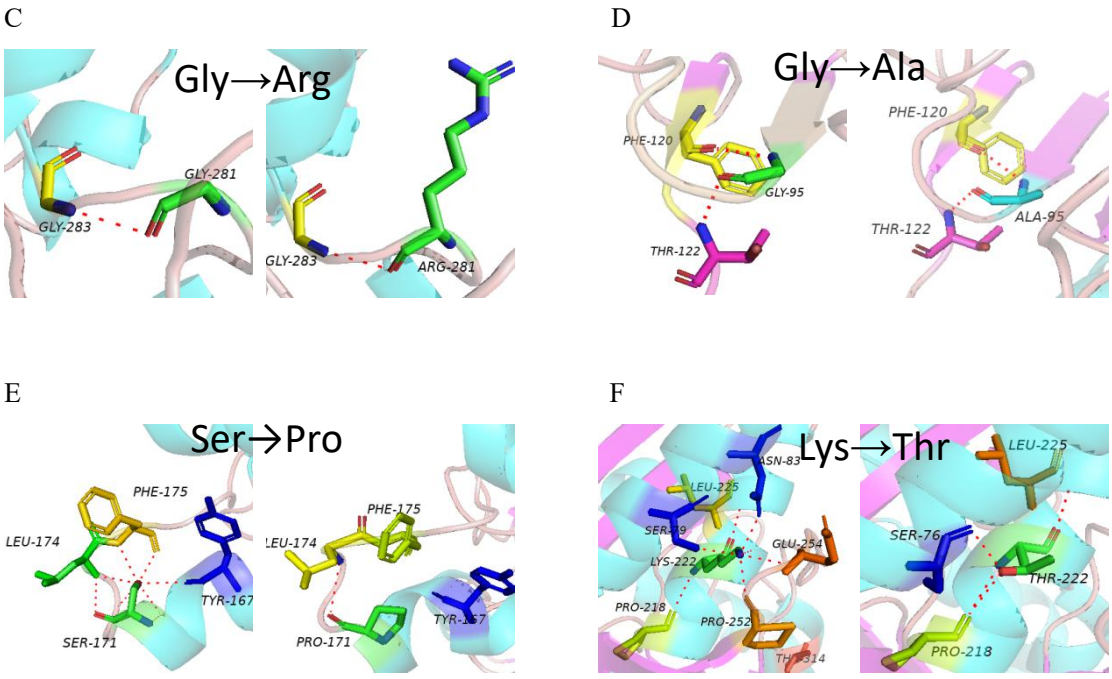

Figure 2: Predicted protein structures of four novel variants in *BCKDHA* and *BCKDHB* genes: p.Gly281Arg(C) in *BCKDHA* gene, p.Gly95Ala(D), p.Ser171Pro(E) and p.Lys222Thr(F) in *BCKDHB* gene

Discussion

Maple syrup urine diabetes is a branched-chain amino acid metabolism disease caused by mitochondrial branched-chain α-keto acid dehydrogenase (BCKDC) deficiency. Scaini *et al.*[9] suggested that cognitive impairment after accumulation of branched-chain amino acids is mainly due to oxidative damage to the brain. The clinical manifestations of MSUD are lack of specificity with rapid onset. The detection of amino acid levels and the ratio between related amino acids in hemofilter paper by tandem mass spectrometry[10] allow for early screening of MSUD and provide an important basis for further diagnosis and treatment. In this study, the results of blood tandem mass spectrometry in all families showed that both leucine and valine were significantly higher, accompanied by amino acid ratio changes, consistent with MSUD biochemical findings.

Gene mutations of MSUD patients are mainly concentrated in the *BCKDHB* gene, followed by *BCKDHA* and *DBT* genes[11]. Current studies suggest that patients with *BCKDHA* and *BCKDHB* gene mutations mostly present with classical type, BCKDH activity is less than 2%. *DBT* gene variants accounts for about 24%, and most of them are thiamine effective type[12]. The clinical manifestations of patients are relatively mild, including developmental retardation and hypotonia.

DLD gene variants account for 13%[13]. Each gene has high allelic heterogeneity, with the exception of gene mutation hotspots found in minority of ethnic groups, such as the most common mutation in the Mennonite community being the *BCKDHA* gene c.1312T>A (p.Tyr393Asn)[14], Portuguese gypsy mutation hotspot c.117delC[15]. The *BCKDHB* gene c.538G > C was a common mutation found in Ashkenazi Jews[16], and exon 5 of the *BCKDHB* gene may be a region of genetic variation and a hotspot region[17, 18]. Hotspot mutations are not found in the remaining population[19–21].There were few reports on MSUD gene mutations in the Chinese population[22–24], and no significant hotspot mutations have been identified. In this study, a total of 13 gene variants (15.4% located in *BCKDHA* gene, 76.9% in *BCKDHB* gene, and 7.7% in *DBT* gene) were found in 16 alleles in 8 families, and the mutation frequency of the *BCKDHB* gene was the highest, which was consistent with the results of other studies[25–27]. The *BCKDHB* gene may be a major variant type of MSUD in the Chinese population. Four of these were novel variants, one located in *BCKDHA* gene p. Gly281Arg and three located in *BCKDHB* gene p. Gly95Ala, p.Lys222Thr, p.Ser171Pro illustrates that the disease has high allelic heterogeneity.

The severity of the MSUD metabolic phenotype is determined by the amount of residual BCKD enzyme activity. The relationship between MSUD genotype and phenotype has not yet been established. The incidence of the disease is low, and fewer cases are included in each study, making it difficult to obtain an exact genotype-phenotype relationship. Patients with nonsense mutations presented the severe classic phenotype. Mutations in p.Arg111Ter and p.Arg285Ter in *BCKDHB* gene generate premature termination codons and the encoded protein has serious effects on the activity of the complex[28]. Our cases in family 5 and 6 carry the nonsense mutation p.Arg111Ter and p.Arg285Ter, respectively, and they have classic phenotype. However, the same type of genetic variation leads to different clinical phenotypes. For example, In *BCKDHA* gene, p.Glu327Lys has been reported to be associated with intermediate phenotype[8] while the same mutation results in classic phenotype in patients in another study[18]. In our study, patient 3 carries the missense mutation p.Arg183Trp in the *BCKDHB* gene and show intermediate phenotype, while the patient who had the same mutation showed classic phenotype in a previous report[29] . Therefore, we could not establish any genotype-phenotype correlation in our patients with MSUD. Half of our cases are classical phenotypes and half are intermediate phenotypes. Majority of

patients with intermediate phenotype had mutations in the *BCKDHB* gene. All the three genes are implicated in classic phenotype. All patients with classic phenotype have worst clinical outcome.

MSUD is a fatal and disabling inherited metabolic disease which is difficult to treat, and has a poor prognosis. Untreated classical children mostly die shortly after birth[29]. Currently, the best preventive strategy for the disease is to avoid the birth of affected children through prenatal diagnosis. However, at the time of presentation of the disease family in clinic, the proband may have died, and novel genetic variants that may cause the disease could be found in the parents of the family. When prenatal diagnosis was performed in the next pregnancy of the same family, pretest counseling should be carefully performed regarding the limitations of result interpretation and prognosis prediction, and informed consent should be obtained before further investigation. Additionally, the traditional prenatal diagnosis method by chorionic villi sampling, amniocentesis and cordocentesis has a certain risk of miscarriage. Although non-invasive genetic diagnosis through haplotype linkage analysis has been developed to assist early diagnosis of MSUD[30], technical and logistical problems must be worked out before the strategy can be clinically useful for MSUD. Preimplantation genetic testing for mendelian disorders (PGT-M) is another prevention strategy for avoiding birth of affected children. However, the current method of PGT-M requires the specimen of the proband for linkage analysis, while the specimen of children with MSUD cannot be obtained because of early death, which greatly limits the clinical application of PGT-M. By contrast, neonatal screening is the key to early diagnosis, and striving for the timing of treatment is an important factor to improve the prognosis of maple syrup diabetes[31]. When MUSD is clinically suspected, capture-based high throughput sequencing followed by Sanger sequencing confirmation allows for accurate detection of gene mutations in the causative genes in an effective manner.

In summary, we applied high-throughput sequencing technology based on target gene capture for sequencing of the four genes in the 8 families with MSUD. NGS combined with Sanger sequencing can detect gene mutations in the causative genes in an effective way, providing clinicians with the basis for differential diagnosis, drug treatment, subsequent genetic counseling and prenatal diagnosis.

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Competing interests The author(s) declare no competing interests.

References:

[1] Miryounesi, M., Ghafouri-Fard, S., Goodarzi, H. & Fardaei, M., A new missense mutation in the BCKDHB gene causes the classic form of maple syrup urine disease (MSUD). *J Pediatr Endocrinol Metab* **28** 673 (2015).

[2] Couce, M. L. *et al.*, Evolution of maple syrup urine disease in patients diagnosed by newborn screening versus late diagnosis. *Eur J Paediatr Neurol* **19** 652 (2015).

[3] Pode-Shakked, N. *et al.*, Clues and challenges in the diagnosis of intermittent maple syrup urine disease. *EUR J MED GENET* **63** 103901 (2020).

[4] Axler, O. & Holmquist, P., Intermittent maple syrup urine disease: two case reports. *PEDIATRICS* **133** e458 (2014).

[5] Scriver, C. R., Clow, C. L. & George, H., So-called thiamin-responsive maple syrup urine disease: 15-year follow-up of the original patient. *The Journal of pediatrics* **107** 763 (1985).

[6] Mitsubuchi, H., Owada, M. & Endo, F., Markers associated with inborn errors of metabolism of branched-chain amino acids and their relevance to upper levels of intake in healthy people: an implication from clinical and molecular investigations on maple syrup urine disease. *J NUTR* **135** 1565S (2005).

[7] Nellis, M. M. *et al.*, Relationship of causative genetic mutations in maple syrup urine disease with their clinical expression. *MOL GENET METAB* **80** 189 (2003).

[8] Rodriguez-Pombo, P., Navarrete, R., Merinero, B., Gomez-Puertas, P. & Ugarte, M., Mutational spectrum of maple syrup urine disease in Spain. *HUM MUTAT* **27** 715 (2006).

[9] Scaini, G. *et al.*, Antioxidant administration prevents memory impairment in an animal model of maple syrup urine disease. *BEHAV BRAIN RES* **231** 92 (2012).

[10] Chace, D. H. *et al.*, Rapid diagnosis of maple syrup urine disease in blood spots from newborns by tandem mass spectrometry. *CLIN CHEM* **41** 62 (1995).

[11] Ali, E. Z. & Ngu, L., Fourteen new mutations of BCKDHA, BCKDHB and DBT genes associated with maple syrup urine disease (MSUD) in Malaysian population. *Molecular Genetics and Metabolism Reports* **17** 22 (2018).

[12] Chuang, D. T., Chuang, J. L. & Wynn, R. M., Lessons from genetic disorders of branched-chain amino acid metabolism. *J NUTR* **136** 243S (2006).

[13] Georgiou, T. *et al.*, Maple syrup urine disease in Cypriot families: identification of three novel mutations and biochemical characterization of the p.Thr211Met mutation in the E1alpha subunit. *Genet Test Mol Biomarkers* **13** 657 (2009).

[14] Love-Gregory, L. D., Grasela, J., Hillman, R. E. & Phillips, C. L., Evidence of common ancestry for the maple syrup urine disease (MSUD) Y438N allele in non-Mennonite MSUD patients. *MOL GENET METAB* **75** 79 (2002).

[15] Quental, S. *et al.*, Revisiting MSUD in Portuguese Gypsies: evidence for a founder mutation and for a mutational hotspot within the BCKDHA gene. *ANN HUM GENET* **73** 298 (2009).

[16] Edelmann, L. *et al.*, Maple syrup urine disease: identification and carrier-frequency determination of a novel founder mutation in the Ashkenazi Jewish population. *AM J HUM GENET* **69** 863 (2001).

[17] Su, L. *et al.*, Two homozygous mutations in the exon 5 of BCKDHB gene that may cause the classic form of maple syrup urine disease. *METAB BRAIN DIS* **32** 765 (2017).

[18] Gupta, D. *et al.*, Identification of mutations, genotype-phenotype correlation and prenatal diagnosis of maple syrup urine disease in Indian patients. *EUR J MED GENET* **58** 471 (2015).

[19] Abiri, M. *et al.*, Identification of six novel mutations in Iranian patients with maple syrup urine disease and their in silico analysis. *Mutat Res* **786** 34 (2016).

[20] Imtiaz, F. *et al.*, Twenty novel mutations in BCKDHA, BCKDHB and DBT genes in a cohort of 52 Saudi Arabian patients with maple syrup urine disease. *Mol Genet Metab Rep* **11** 17 (2017).

[21] Xiyuan Li, et al. Clinical, biochemical and genetic features of 13 children with maple syrup urine disease. Chinese Journal of Applied Clinical Pediatrics. **31**, 569-572 (2016).

[22] Yang, N. *et al.*, Analysis of gene mutations in Chinese patients with maple syrup urine disease. *MOL GENET METAB* **106** 412 (2012).

[23] Li, X. *et al.*, Eleven novel mutations of the BCKDHA, BCKDHB and DBT genes associated with maple syrup urine disease in the Chinese population: Report on eight cases. *EUR J MED GENET* **58** 617 (2015).

[24] Li, W. *et al.*, Silico analysis of a novel mutation c.550delT in a Chinese patient with maple syrup urine disease. *Clin Case Rep* **6** 1989 (2018).

[25] Yang, C., Linpeng, S., Cao, Y. & Wu, L., Identification of six novel mutations in five infants with suspected maple syrup urine disease based on blood and urine metabolism screening. *GENE* **710** 9 (2019).

[26] Li, X. *et al.*, Clinical characteristics and mutation analysis of five Chinese patients with maple syrup urine disease. *METAB BRAIN DIS* **33** 741 (2018).

[27] Sun, W. *et al.*, Identification of eight novel mutations in 11 Chinese patients with maple syrup urine disease. *WORLD J PEDIATR* (2020).

[28] Flaschker, N. *et al.*, Description of the mutations in 15 subjects with variant forms of maple syrup urine disease. *J INHERIT METAB DIS* **30** 903 (2007).

[29] Abiri, M. *et al.*, Maple syrup urine disease mutation spectrum in a cohort of 40 consanguineous patients and insilico analysis of novel mutations. *METAB BRAIN DIS* **34** 1145 (2019).

[30] You, Y. *et al.*, Integration of targeted sequencing and NIPT into clinical practice in a Chinese family with maple syrup urine disease. *GENET MED* **16** 594 (2014).

[31] Strauss, K. A. *et al.*, Classical maple syrup urine disease and brain development: principles of management and formula design. *MOL GENET METAB* **99** 333 (2010).

Figures

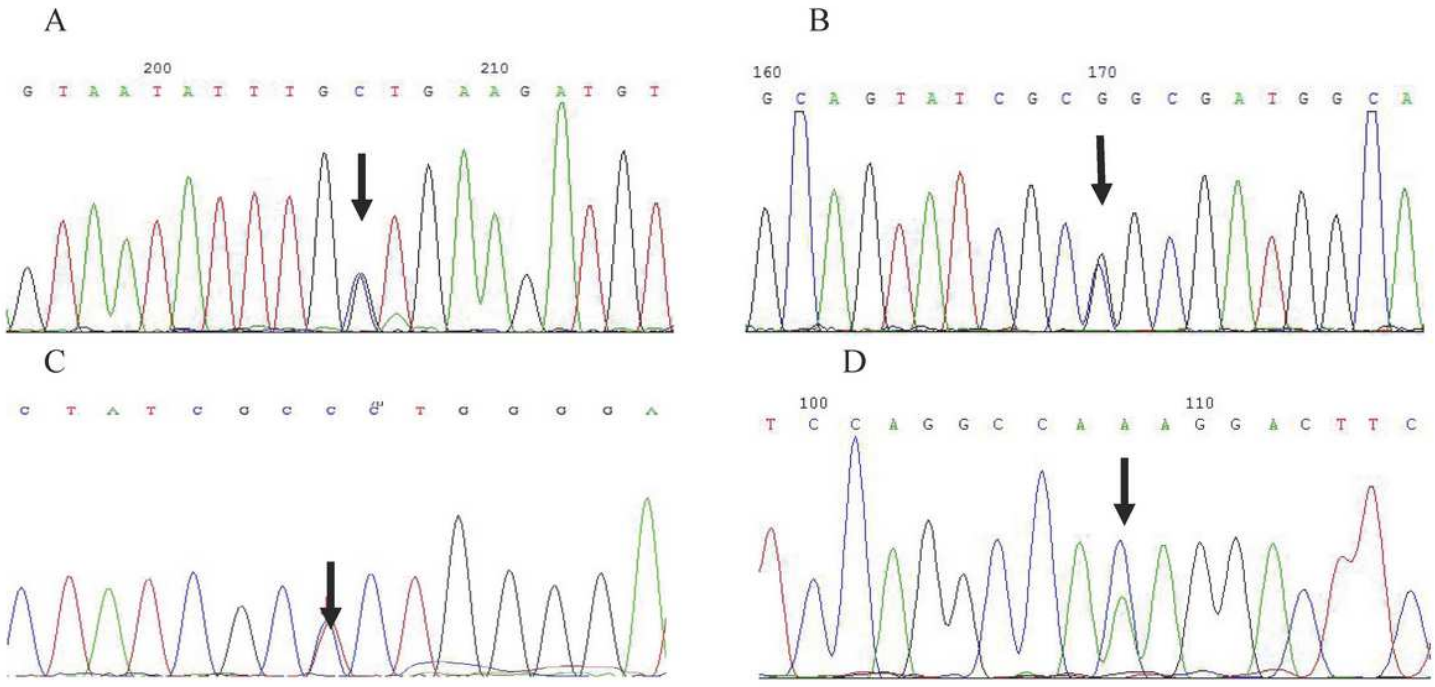

Figure 1

Gene sequences of four novel variant in BCKDHA and BCKDHB genes in eight pedigrees: c.841G>C(p.Gly281Arg) in BCKDHA gene(A), c.284G>C(p.Gly95Ala) in BCKDHB gene(B), c.511T>C(p.Ser171Pro) in BCKDHB gene(C), c.665A>C(p.Lys222Thr) in BCKDHB gene (D)

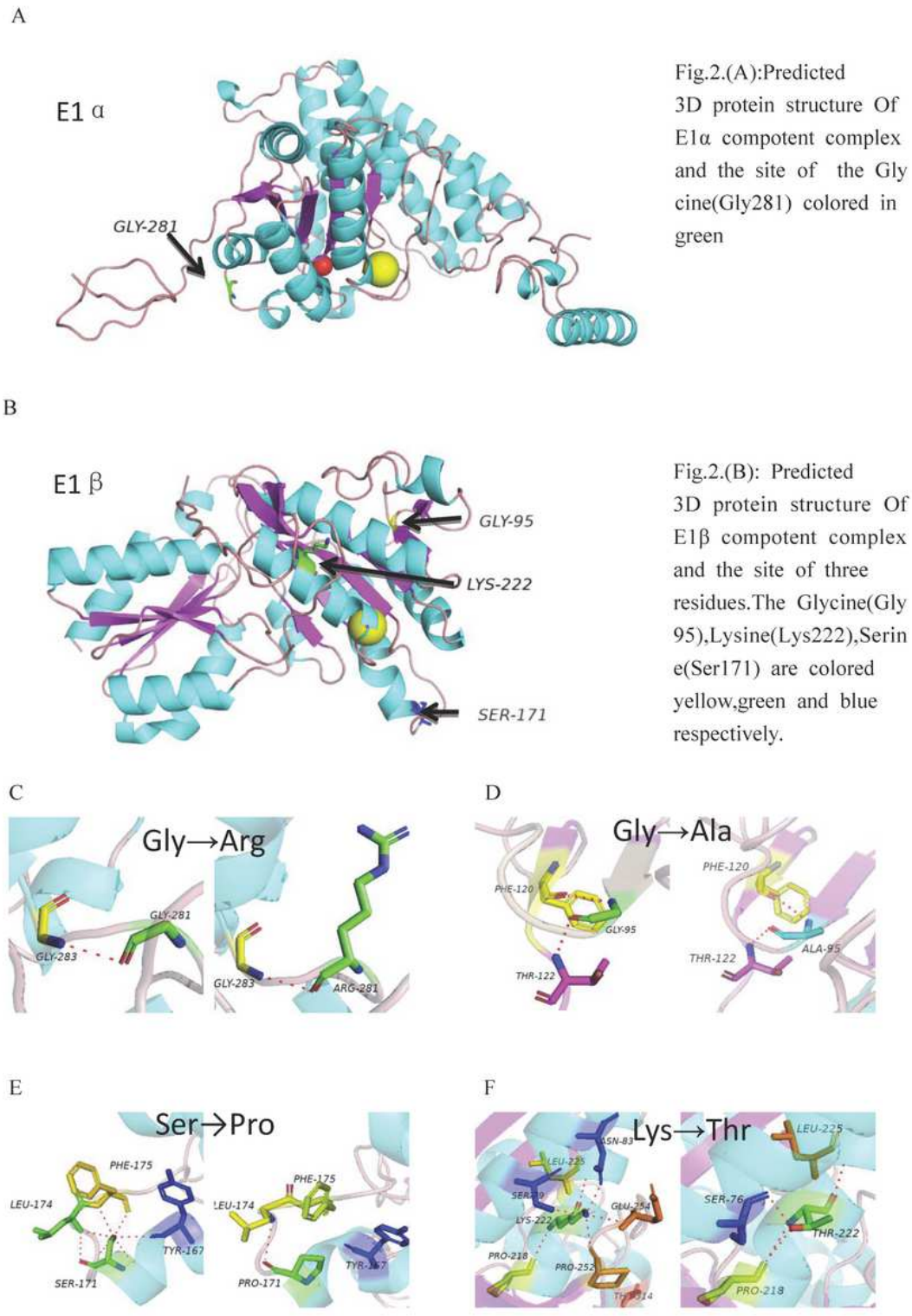

Figure 2

Predicted protein structures of four novel variants in BCKDHA and BCKDHB genes: p.Gly281Arg(C) in BCKDHA gene, p.Gly95Ala(D), p.Ser171Pro(E) and p.Lys222Thr(F) in BCKDHB gene

Supplementary Files

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- Table.pdf