Pathology - Research and Practice 215 (2019) 152702

Contents lists available at ScienceDirect

Pathology - Research and Practice

journal homepage: www.elsevier.com/locate/prp

Expression and significance of ETFDH in hepatocellular carcinoma

Yaxun Wu[1], Xingsong Zhang[1], Rong Shen, Jieyu Huang, Xiaoyun Lu, Guihua Zheng*, Xudong Chen*

Department of Pathology, Affiliated Tumor Hospital of Nantong University, Nantong 226361, Jiangsu, China

ARTICLE INFO

Keywords:
Hepatocellular carcinoma
ETFDH
Immunohistochemistry
Survival

ABSTRACT

The *ETFDH* (electron transfer flavoprotein dehydrogenase) gene mutations are reported to be a major cause of riboflavin-responsive multiple acyl-coenzyme A dehydrogenation deficiency (MADD). However, the role of ETFDH in the prognosis of hepatocellular carcinoma (HCC) remains unclear. The aim of this study was to investigate the expression of ETFDH in HCC. Immunohistochemical staining of the 207 HCC tissue microarray showed that expression of ETFDH was significantly decreased in HCC compared with the matching noncancerous hepatic tissues ($P < 0.001$). Moreover, ETFDH expression levels were found to be correlated with AFP levels ($P = 0.011$). Intriguingly, ETFDH expression levels were significantly lower in poorly differentiated or undifferentiated HCCs as compared to the well or moderately differentiated cases ($P = 0.001$). Kaplan-Meier analysis revealed that low tumor expression of ETFDH was associated with a poorer overall survival in patients with HCC ($P = 0.024$). Furthermore, multivariate analysis showed that ETFDH ($P = 0.047$) was an independent predictor of overall survival. Our findings may shed new light on the identification of new prognostic marker for HCC.

1. Introduction

Liver cancer is the fourth leading cause of cancer death worldwide in 2018 [1]. Hepatocellular carcinoma (HCC) is the most prevalent primary liver cancer, accounting for 80–90% of all primary liver cancer in many countries [2]. Despite marked progress in therapeutic strategies, the prognosis for HCC patients remains poor [3,4]. Therefore, identification of the underlying molecular mechanisms in HCC pathogenesis may contribute to a better clinical management of such patients.

The *ETFDH* (electron transfer flavoprotein dehydrogenase) gene (also referred to as electron-transfer flavoprotein ubiquinone oxidoreductase, ETF-QO), is located in 4q32.1 and contains 13 exons [5]. It comprises three functional domains: a FAD domain, an iron-sulfur [4Fe4S] cluster domain, and a ubiquinone (UQ) binding domain [6]. The ETF (electron transfer flavoprotein) and ETFDH link the oxidation of fatty acids and some amino acids to the electron transport chain by transferring electrons from acyl-CoA dehydrogenases to ubiquinone in the inner mitochondrial membrane [7]. Previous data have shown that many missense mutations in ETFDH impair FAD binding. FAD has a crucial role in maintaining conformational stability and the proper folding of lots of flavoproteins. Increased FAD concentration is reported to restore stability of ETFDH proteins carrying missense mutations [8]. *ETFDH* gene mutations are reported to be an important cause of riboflavin-responsive multiple acyl-coenzyme A dehydrogenation deficiency (MADD) [9]. The appearance of clinical symptoms of MADD are heterogeneous, however, ETFDH mutations usually lead to a lateronset milder phenotype, which mainly manifested as episodic weakness, exercise intolerance, rhabdomyolysis, hepatomegaly, gastrointestinal symptoms, and weight loss [10]. To date, the limited literature on ETFDH has focused primarily on MADD. However, the role of ETFDH in the prognosis of HCC remains unclear.

In the present study, we investigated the correlation between the expression of ETFDH and the prognosis of HCC. Our study provides evidence that ETFDH may serve as a prognostic and predictive biomarker in HCC.

2. Materials and methods

2.1. Patients and specimens

The patient cohort consists of 207 patients with HCC who had been surgically treated in the Affiliated Tumor Hospital of Nantong University from January 2009 to January 2014. The inclusion criteria

* Corresponding authors.
E-mail addresses: zhengguihua1999@163.com (G. Zheng), chenxudong@ntu.edu.cn (X. Chen).
[1] Yaxun Wu and Xingsong Zhang have contributed equally to this work.

https://doi.org/10.1016/j.prp.2019.152702
Received 7 July 2019; Received in revised form 6 October 2019; Accepted 19 October 2019

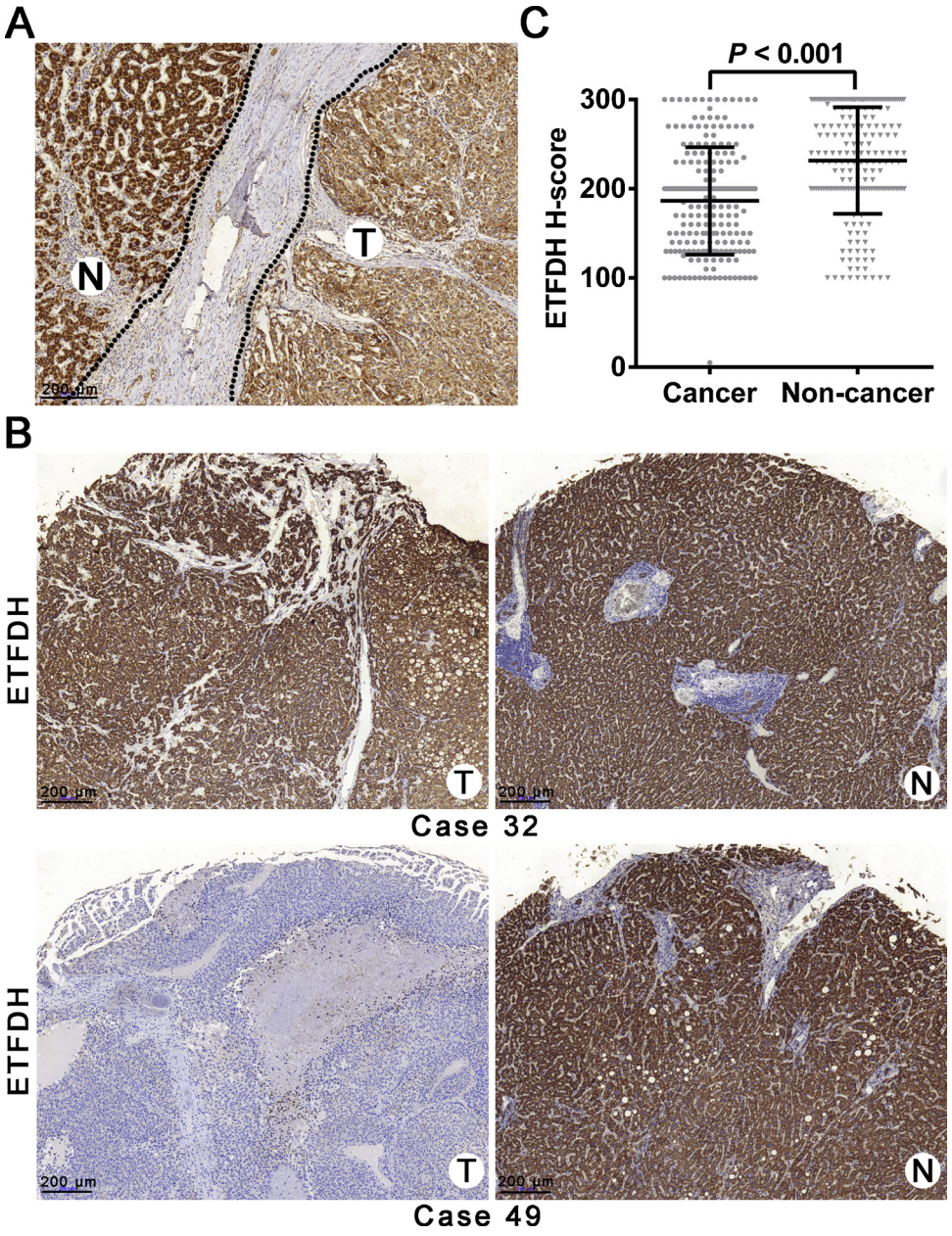

Fig. 1. ETFDH is frequently decreased in HCC. (A) Representative image of immunohistochemical staining of the whole tissue section with anti-ETFDH antibody. (B) Immunohistochemical staining of HCC TMAs and the matching noncancerous hepatic tissues. Shown are stained tumor and noncancerous hepatic tissue sections representative of high and low ETFDH staining. (C) Scatter plots showing a statistical analysis of ETFDH expression in HCC and the matching noncancerous hepatic tissues.

were: (1) pathologic confirmation of HCC; (2) without a history of cancer; (3) not receiving any treatment prior to surgery. The exclusion criteria were: (1) underwent liver transplantation during follow-up; (2) other causes of death. The median age of the patients was 51 years (range = 35–75 years), and the median follow-up was 57 months (range = 1–104 months), 99 (47.8%) patients died during follow-up. Written informed consent was obtained from each patient. Ethical permission was received from the Ethical Review Committee of the Affiliated Tumor Hospital of Nantong University. After ethical approval, 207 HCC samples and corresponding noncancerous hepatic tissues were obtained to construct tissue array (TMA) as previously described [11].

2.2. Immunohistochemistry

Immunohistochemical staining was performed on a Dako Omnis Autostainer (Dako, Agilent Technologies, Inc., Carpinteria, CA, USA) according to the manufacturer's instructions. Briefly, sections were baked at 60 °C for 30 min, and then loaded onto the Dako Omnis Autostainer for deparaffinization, antigen retrieval, antibody incubation and detection. Antigen retrieval was performed using EnVision™ FLEX Target Retrieval Solution, high pH at 97 °C for 30 min. ETFDH antibody (1:200 dilution, Proteintech Group, Wuhan, China) was incubated for 28 min. Detection was performed using the EnVision FLEX/HRP reagent for 30 min.

Table 1
Relationship between expression levels of ETFDH and clinicopathological features of 207 HCC specimens.

Variables	n	ETFDH		*P* value
		Low	High	
Age (years)				0.811
< 60	146	21	125	
≥60	61	8	53	
Gender				0.670
Female	37	6	31	
Male	170	23	147	
Tumor size				0.364
< 5 cm	116	14	102	
≥5 cm	91	15	76	
AFP				0.011
< 400 ng/mL	136	13	123	
≥400 ng/mL	71	16	55	
HBsAg				0.118
Negative	36	8	28	
Positive	171	21	150	
Tumor number				0.709
Single	166	24	142	
Multiple	41	5	36	
Liver cirrhosis				0.442
Absent	59	10	49	
Present	148	19	129	
Vascular invasion				0.212
Absent	142	17	125	
Present	65	12	53	
TNM				0.276
I / II	126	15	111	
III / IV	81	14	67	
Edmonson grade				0.001
I - II	128	10	118	
III - IV	79	19	60	

Statistical analyses were performed by the Pearson χ^2 test.
A P value < 0.05 was considered significant.

2.3. Evaluation of immunohistochemistry

For the quantitative analysis, the H-score was calculated based on the staining intensity and percentage of stained cells. The intensity score was defined as follows: 0, negative; 1, weak staining; 2, intermediate staining; 3, strong staining. The fraction of positive cells was scored as 0–100%. The H-score was calculated by multiplying the intensity score and the fraction score, producing a total range of 0 - 300. The optimal cutoff point for the definition of ETFDH high/low expression subgroups was calculated by X-tile software (Yale University). Cases exhibiting a H-score 0–120 were pooled in the ETFDH low expression group, whereas cases with a higher H-score (> 120) were designated ETFDH high expression group.

2.4. Statistical analysis

The associations between ETFDH expression and clinicopathological characteristics were evaluated by Pearson's chi-square test. Kaplan-Meier analysis and the log-rank test was used to estimate differences in overall survival. Cox proportional-hazards regression analysis was applied to estimate univariate and multivariate hazard ratios for overall survival. *P*-values < 0.05 were considered significant.

3. Results

3.1. Reduced ETFDH expression in HCC versus noncancerous hepatic tissues

From our preliminary experiments, we observed that expression of ETFDH was significantly decreased in HCC cells in whole tissue sections (Fig. 1A). These results were confirmed in TMA, which showed that ETFDH staining was much weaker in HCC (H-score, 186.4 ± 4.178) than the matching noncancerous hepatic tissues (H-score, 231.7 ± 4.150) ($P < 0.001$; Fig. 1B and 1C). As shown in Table 1, ETFDH expression levels were found to be correlated with AFP levels ($P = 0.011$). Of the 128 Edmondson grade I or II tumors, 118 (92.2%) had high ETFDH expression and 10 (7.8%) had low ETFDH expression. Of the 79 Edmondson grade III or IV tumors, 60 (75.9%) had high ETFDH expression and 19 (24.1%) had low ETFDH expression ($P = 0.001$; Table 1). These findings suggested that ETFDH expression levels were significantly lower in poorly differentiated or undifferentiated HCCs as compared to the well or moderately differentiated cases. However, ETFDH expression was not correlated with age, gender, tumor size, HBsAg status, tumor number, liver cirrhosis, vascular invasion and TNM stage ($P > 0.05$ for all; Table 1).

Table 2
Univariate and multivariate analysis of prognostic factors in 207 HCC patients.

	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P*	HR (95% CI)	*P*
Age				
< 60 years	1.00			
≥60 years	1.076 (0.701 to 1.654)	0.737		
Gender				
Female	1.00			
Male	0.911 (0.546 to 1.519)	0.721		
Tumor size				
< 5 cm	1.00		1.00	
≥5 cm	1.610 (1.085 to 2.390)	0.018	0.673 (0.282 to 1.609)	0.373
AFP				
< 400 ng/mL	1.00			
≥400 ng/mL	1.341 (0.892 to 2.016)	0.158		
HBsAg				
Negative	1.00			
Positive	1.491 (0.831 to 2.675)	0.181		
Tumor number				
Single	1.00		1.00	
Multiple	1.609 (1.013 to 2.554)	0.044	1.539 (0.943 to 2.513)	0.085
Liver cirrhosis				
Absent	1.00			
Present	1.559 (0.970 to 2.505)	0.066		
Vascular invasion				
Absent	1.00		1.00	
Present	1.516 (1.006 to 2.286)	0.047	0.983 (0.609 to 1.586)	0.943
TNM				
I / II	1.00		1.00	
III / IV	2.017 (1.358 to 2.997)	0.001	2.759 (1.139 to 6.685)	0.025
Edmonson grade				
I - II	1.00			
III - IV	1.130 (0.753 to 1.695)	0.554		
ETFDH				
Low	1.00		1.00	
High	0.560 (0.336 to 0.934)	0.026	0.589 (0.350 to 0.992)	0.047

Statistical analyses were performed by Cox proportional hazards regression.
A P value < 0.05 was considered significant.

3.2. Low ETFDH expression predicts poor overall survival in HCC

Cox univariate analysis suggested that decreased ETFDH expression was associated with reduced overall survival time (HR = 0.560, 95% CI = 0.336 to 0.934, $P = 0.026$; Table 2). The association between ETFDH expression and overall survival time was confirmed by Kaplan-

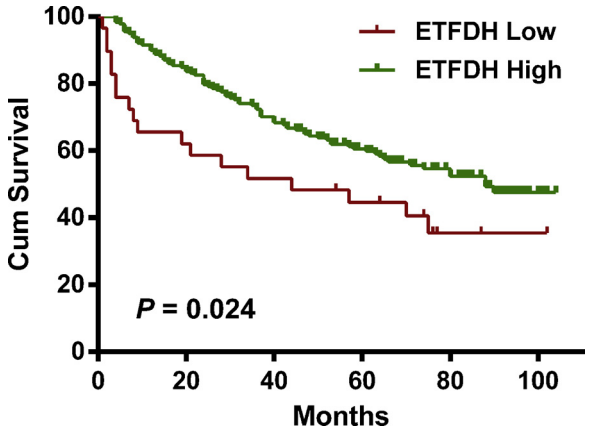

Fig. 2. Kaplan-Meier survival curves for 207 HCC patients according to ETFDH expression status (log-rank test, $P < 0.024$).

Meier analysis, which showed that low tumor expression of ETFDH was associated with a poorer overall survival in patients with HCC ($P = 0.024$; Fig. 2). Furthermore, in univariate analysis, tumor size ($P = 0.018$), tumor number ($P = 0.044$), vascular invasion ($P = 0.047$), and TNM stage ($P = 0.001$) showed a significant impact on survival, whereas age ($P = 0.737$), gender ($P = 0.721$), AFP level ($P = 0.158$), HBsAg status ($P = 0.181$), liver cirrhosis ($P = 0.066$), and Edmonson grade ($P = 0.554$) were not significantly associated with survival (Table 2). Intriguingly, multivariate analysis demonstrated that TNM stage ($P = 0.025$) and ETFDH ($P = 0.047$) were independent predictors of overall survival (Table 2).

4. Discussion

Physiologically, the first step in the mitochondrial beta-oxidation process is the acyl group CoA dehydrogenase catalyzed fatty acid dehydrogenation reaction. During the beta-oxidation of lipophilic acid, electrons produced by multiple acyl-coenzyme A dehydrogenase are transferred to ETF, and then transferred to ETFDH located in the mitochondrial membrane, and transferred to the respiratory chain complex III via ETFDH-bound ubiquinone pool, resulting in ATP as body energy supply [6]. ETF is a heterodimer of alpha (30 kDa) and beta (28 kDa) subunits, encoded by *ETFA* and *ETFB* genes, respectively. It contains a flavin adenine dinucleotide (FAD) prosthesis and an adenosine 5′-monophosphate in the mitochondrial matrix [12]. The genetic defects of ETFDH or ETF lead to metabolic diseases, MADD, also known as glutaric acidemia type II (GAII) [13–15]. MADD is an autosomal recessive genetic disorder with mitochondrial electron transport chains and metabolic disorders of fatty acids [8,16,17]. MADD shows a broad range of symptoms, including hypoglycemia, recurrent rhabdomyolysis, cardiomyopathy, encephalopathy, and lipid storage myopathy. The phenotypes of MADD are divided into three categories: a neonatal-onset form with congenital anomalies (type I) or without congenital anomalies (type II) and a mild and/or late-onset form (type III) [18–20].

To date, the limited literature on ETFDH has focused primarily on MADD. However, the role of ETFDH in the prognosis of HCC remains unclear. In this study, we found that expression of ETFDH was significantly decreased in HCC compared with the matching noncancerous hepatic tissues. Moreover, ETFDH expression levels were found to be correlated with AFP levels. Intriguingly, we found that ETFDH expression levels were significantly lower in poorly differentiated or undifferentiated HCCs as compared to the well or moderately differentiated cases. Kaplan-Meier analysis revealed that low tumor expression of ETFDH was associated with a poorer overall survival in patients with HCC ($P = 0.024$). Furthermore, multivariate analysis showed that ETFDH ($P = 0.047$) was an independent predictor of overall survival.

In conclusion, the current study demonstrated that low tumor expression of ETFDH was associated with a poorer overall survival in patients with HCC, and ETFDH was an independent predictor of overall survival. Our findings may shed new light on the identification of new prognostic marker for HCC.

Conflict of interest statement

None.

Acknowledgments

This work was supported by grants from the Nantong Science and Technology Project Foundation (JC2018033).

References

[1] F. Bray, J. Ferlay, I. Soerjomataram, R.L. Siegel, L.A. Torre, A. Jemal, Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries, CA Cancer J. Clin. 68 (2018) 394–424.
[2] M. Ringelhan, D. Pfister, T. O'Connor, E. Pikarsky, M. Heikenwalder, The immunology of hepatocellular carcinoma, Nat. Immunol. 19 (2018) 222–232.
[3] T. Lan, K. Yuan, X. Yan, L. Xu, H. Liao, X. Hao, et al., LncRNA SNHG10 facilitates hepatocarcinogenesis and metastasis by modulating its homolog SCARNA13 via a positive feedback loop, Cancer Res. (2019).
[4] D. Galun, A. Bogdanovic, J. Djokic Kovac, P. Bulajic, Z. Loncar, M. Zuvela, Preoperative neutrophil-to-lymphocyte ratio as a prognostic predictor after curative-intent surgery for hepatocellular carcinoma: experience from a developing country, Cancer Manag. Res. 10 (2018) 977–988.
[5] Y. Xue, Y. Zhou, K. Zhang, L. Li, A. Kayoumu, L. Chen, et al., Compound heterozygous mutations in electron transfer flavoprotein dehydrogenase identified in a young Chinese woman with late-onset glutaric aciduria type II, Lipids Health Dis. 16 (2017) 185.
[6] J. Zhang, F.E. Frerman, J.J. Kim, Structure of electron transfer flavoprotein-ubiquinone oxidoreductase and electron transfer to the mitochondrial ubiquinone pool, Proc Natl Acad Sci U S A. 103 (2006) 16212–16217.
[7] S. Mosegaard, G.H. Bruun, K.F. Flyvbjerg, Y.T. Bliksrud, N. Gregersen, M. Dembic, et al., An intronic variation in SLC52A1 causes exon skipping and transient riboflavin-responsive multiple acyl-CoA dehydrogenation deficiency, Mol. Genet. Metab. 122 (2017) 182–188.
[8] S. Missaglia, D. Tavian, L. Moro, C. Angelini, Characterization of two ETFDH mutations in a novel case of riboflavin-responsive multiple acyl-CoA dehydrogenase deficiency, Lipids Health Dis. 17 (2018) 254.
[9] F. Sugai, K. Baba, K. Toyooka, W.C. Liang, I. Nishino, M. Yamadera, et al., Adult-onset multiple acyl CoA dehydrogenation deficiency associated with an abnormal isoenzyme pattern of serum lactate dehydrogenase, Neuromuscul. Disord. 22 (2012) 159–161.
[10] W.C. Liang, Y.F. Lin, T.Y. Liu, S.C. Chang, B.H. Chen, I. Nishino, et al., Neurite growth could be impaired by ETFDH mutation but restored by mitochondrial cofactors, Muscle Nerve 56 (2017) 479–485.
[11] L. Song, D. Liu, X. Zhang, X. Zhu, X. Lu, J. Huang, et al., Low expression of PDHA1 predicts poor prognosis in gastric cancer, Pathol. Res. Pract. 215 (2019) 478–482.
[12] R.K. Olsen, B.S. Andresen, E. Christensen, P. Bross, F. Skovby, N. Gregersen, Clear relationship between ETF/ETFDH genotype and phenotype in patients with multiple acyl-CoA dehydrogenation deficiency, Hum. Mutat. 22 (2003) 12–23.
[13] S.I. Goodman, R.J. Binard, M.R. Woontner, F.E. Frerman, Glutaric acidemia type II: gene structure and mutations of the electron transfer flavoprotein:ubiquinone oxidoreductase (ETF:QO) gene, Mol. Genet. Metab. 77 (2002) 86–90.
[14] L.L. Goh, Y. Lee, E.S. Tan, J.S.C. Lim, C.W. Lim, R. Dalan, Patient with multiple acyl-CoA dehydrogenase deficiency disease and ETFDH mutations benefits from riboflavin therapy: a case report, BMC Med. Genomics 11 (2018) 37.
[15] J. Xi, B. Wen, J. Lin, W. Zhu, S. Luo, C. Zhao, et al., Clinical features and ETFDH mutation spectrum in a cohort of 90 Chinese patients with late-onset multiple acyl-CoA dehydrogenase deficiency, J. Inherit. Metab. Dis. 37 (2014) 399–404.
[16] Z. Zhuo, P. Jin, F. Li, H. Li, X. Chen, H. Wang, A case of late-onset riboflavin responsive multiple acyl-CoA dehydrogenase deficiency (MADD) with a novel mutation in ETFDH gene, J. Neurol. Sci. 353 (2015) 84–86.
[17] J. Xu, D. Li, J. Lv, X. Xu, B. Wen, P. Lin, et al., ETFDH Mutations and Flavin Adenine Dinucleotide Homeostasis Disturbance Are Essential for Developing Riboflavin-Responsive Multiple Acyl-Coenzyme A Dehydrogenation Deficiency, Ann. Neurol. 84 (2018) 659–673.
[18] E.M. Pennisi, M. Garibaldi, G. Antonini, Lipid Myopathies, J. Clin. Med. 7 (2018).
[19] H.Z. Chen, M. Jin, N.Q. Cai, X.D. Lin, X.Y. Liu, L.Q. Xu, et al., Rhabdomyolysis and respiratory insufficiency due to the common ETFDH mutation of c.250G&A in two patients with late-onset multiple acyl-CoA dehydrogenase deficiency, Chin Med J (Engl). 132 (2019) 1615–1618.
[20] X. Fan, B. Xie, J. Zou, J. Luo, Z. Qin, A.M. D'Gama, et al., Novel ETFDH mutations in four cases of riboflavin responsive multiple acyl-CoA dehydrogenase deficiency, Mol. Genet. Metab. Rep. 16 (2018) 15–19.