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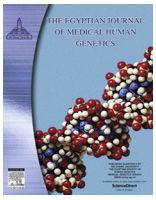

Case Report

Two Libyan siblings with beta-ketothiolase deficiency: A case report and review of literature

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ABSTRACT

Beta-ketothiolase (mitochondrial acetoacetyl-CoA thiolase, T2) deficiency is an autosomal recessive disorder characterized by impaired metabolism of ketones and isoleucine. In this study, we report on the first two siblings with T2 deficiency from Libya. Both siblings presented with ketoacidosis, but the severity and outcomes were quite distinctive. T2 deficiency in patient 1, the younger sister, manifested as recurrent severe episodes of ketoacidosis during the first year of life. She unfortunately experienced neurodevelopmental complications, and died at 14 months old, after her 5th episode. In contrast, patient 2, the elder brother, experienced only one ketoacidotic episode at the age of 4 years. He recovered uneventfully and has continued to achieve age-appropriate development to date. Upon analysis, the siblings' blood acylcarnitine profiles had shown increased levels of C5:1 and C5-OH carnitine. *ACAT1* mutational analysis revealed patient 2 is homozygotic for a novel mutation–c.674C > A (p.Ala225Glu); this mutation was then confirmed by familial analysis. Transient expression analysis of c.674C > A mutant T2 cDNA revealed neither potassium ion-activated acetoacetyl-CoA thiolase activity, which represents T2 activity, nor mutant T2 protein. Therefore, this mutation is truly pathogenic. Interestingly, the incidence of T2 deficiency may be high among the Arab population. This disease should be considered in the differential diagnosis for unexplained ketoacidosis in children. Patients with T2 deficiency could have a favorable outcome if diagnosed and treated early.

1. Introduction

Beta-ketothiolase deficiency (Online Mendelian Inheritance in Man [OMIM] 203750, 607809), a defect in mitochondrial acetoacetyl-CoA thiolase (T2) (EC 2.3.1.9, gene symbol *ACAT1*), is an autosomal recessive disease that affects isoleucine catabolism and ketone body utilization [1,2]. Since Daum et al. first characterized T2 deficiency in 1971, more than 100 patients have been diagnosed with the disease worldwide [3]. T2 deficiency ordinarily manifests as intermittent ketoacidotic episodes between the ages of 6 and 18 months; patients are usually asymptomatic between episodes. A distinctive laboratory finding is an increased urinary excretion of isoleucine-catabolic intermediates 2-methyl-3-hydroxybutyrate (2M3HB), 2-methylacetoacetate (2MAA), and tiglylglycine (TIG). In addition, blood acylcarnitine analysis usually shows elevated C5:1 and C5-OH carnitine levels [1,2]. Nevertheless, some T2-deficient patients demonstrated atypical clinical and laboratory features. The severity and frequency of episodes vary among patients; however, the outcome is commonly favorable, unless a ketoacidotic episode causes life-threatening or irreversible sequelae [1,3].

The human *ACAT1* gene is located at chromosome 11q22.3-23.1. It spans nearly 27 kb and contains 12 exons and 11 introns.

Abbreviations: 2MAA, 2-methylacetoacetate; 2M3HB, 2-methyl-3-hydroxybutyrate; MS/MS, tandem mass spectrometry; SCOT, succinyl-CoA:3-oxoacid CoA transferase; T2, mitochondrial acetoacetyl-CoA thiolase; TIG, tiglylglycine; TKB, total ketone body.

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T2 cDNA is approximately 1.5 kb in length and encodes a precursor protein consisting of 427 amino acids [4]. *ACAT1* mutations are highly diverse; more than 70 mutations have been identified to date. Except for p.Arg208* among the Vietnamese, no other common *ACAT1* mutation patterns have yet been recognized [3,5].

There are few studies on T2 deficiency in the Arab population. Several cases have been detected by tandem mass spectrometry (MS/MS), but the diagnoses were seldom confirmed by an enzyme assay or genetic analysis [6–18]. In this study, we report on the first two Libyan siblings, sister and brother, with T2 deficiency. We describe their clinical and laboratory features and characterize a novel *ACAT1* mutation. We also discuss the current situation of T2 deficiency among Arab population and review main characteristics of this disorder, which may point to diagnostic clues.

2. Patients and methods

Patient 1: The female patient was the third of four children born to first cousins parents of Arab ancestry in Libya. She was delivered at full term following an uneventful gestation, weighing 3 kg. There was no prior remarkable family history, as her elder brother, patient 2, was asymptomatic at that time. The patient thrived until 20 days of age when she was admitted to a hospital with vomiting, diarrhea, dehydration, and acidotic breathing. Symptomatic management, including intravenous fluids, was given; she recovered uneventfully. A hydrolyzed milk formula was prescribed for presumed cow's milk protein intolerance. No additional investigation was performed for this episode.

At 7 months of age, the patient was admitted to an intensive care unit with dehydration, acidotic breathing, and lethargy. This was preceded by fever, vomiting, and diarrhea for 2 days. Physical examination revealed hepatomegaly, marked hypotonia, hyporeflexia, and abnormal pupillary response to light, showing no reaction on the right and a sluggish reaction on the left. Arterial blood gases revealed a severe metabolic acidosis: pH 6.8, HCO_3 3 mmol/L, base excess −24, and pCO_2 15 mmHg. Ketonuria was detected. A blood count demonstrated leukocytosis ($31 \times 10^3/\mu l$), mild anemia (9.6 gm/dl), and normal platelets. Other investigations showed a blood glucose of 2.5 mmol/L, lactate 2 mmol/L, and ammonia 145 μmol/L; her electrolytes and liver and kidney function tests revealed no significant abnormalities. Management for an assumed central nervous system infection, such as meningitis or encephalitis, was implemented. After 12 days, her general condition was stabilized. She was then referred to a metabolic unit, where an organic aciduria was suspected. A subsequent acylcarnitine analysis of dried blood spots showed increased C5:1 (0.34 μmol/L; cut-off point 0.12) and C5-OH (2.68 μmol/L; cut-off point 0.47) levels. Precautionary measures, including avoidance of prolonged fasting, protein restriction (1.5 g/kg/day), carnitine supplementation (100 mg/kg/day), and early recognition of illness symptoms, were prescribed.

Follow-up demonstrated a neurodevelopmental delay, irritability, progressive dystonia, choreic movements, and nystagmus. At 12 months of age, a brain magnetic resonance imaging was conducted. This showed cerebral atrophy and bilateral hyperintense areas involving caudate and putamen nuclei on T2-weighed images. An electroencephalogram showed hypsarrhythmia. Two mild ketoacidotic episodes, precipitated by infections, recurred at 9 and 12 months of age. At 14 months of age, the patient experienced her final episode, which was more severe than her 2nd episode. She succumbed to her disease at that time.

Patient 2: The male patient, the elder brother of patient 1, was the firstborn child of his parents. There were no remarkable antenatal events. He was born at full term with a birth weight of 4 kg. He was well until 4 years and 2 months of age when he was admitted to an intensive care unit with vomiting, acidotic breathing, lethargy, and generalized convulsions. This was preceded by fever, sore throat, and poor oral intake for 5 days. Arterial blood gases showed pH 7.11, HCO_3 3.4 mmol/L, base excess −20, and pCO_2 4.3 mmHg. Urinary ketone body testing was positive. Other investigations revealed severe hypoglycemia (0.95 mmol/L), ammonia 144 μmol/L, and lactate 1.67 mmol/L. Patient 2 was provisionally diagnosed with T2 deficiency because his sister (patient 1) had already been diagnosed with this disease one month before. Therefore, a timely blood acylcarnitine analysis was performed. It demonstrated increased C5:1 (0.41 μmol/L) and C5-OH (4.13 μmol/L) levels. He was treated successfully with appropriate intravenous glucose, fluids, bicarbonate, and electrolytes. The previously-described precautionary measures were reinforced. At his current age of 7.5 years, he has not suffered of any further episodes. He has attained age-appropriate growth and development. Recently, dried blood spots were obtained from the living family members for an *ACAT1* mutational analysis.

This study was approved by the Ethical Committee of the Graduate School of Medicine, Gifu University, Japan. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consents were obtained from all participants or their parents for inclusion in the study.

2.1. Mutation analysis

Small pieces (1.2 × 1.2 mm) were used for direct PCR amplification of DNA from dried blood specimens. The 12 *ACAT1* exons, with flanking intron regions, were amplified by primer pairs as described before [19]. PCR conditions for all segments, using a BIOTaq™ HS DNA Polymerase (Bioline Reagents Ltd, London, UK), were 95 °C for 10 min; 40 cycles of 94 °C for 30 s, 55 °C for 1 min, and 72 °C for 1 min; followed by 72 °C for 7 min. The 12 amplified fragments were sequenced using a BigDye® Terminator v1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and ABI PRISM 3130XL Genetic Analyzer (Applied Biosystems) per the manufacturer's directions. The genomic *ACAT1* sequence (GenBank accession NG_009888.1) was used as a reference.

2.2. Transient expression analysis

A pCAGGS eukaryote expression vector was used for transient expression analysis of T2 cDNA, as previously described [19–21]. In brief, we used a Kod-Plus-Mutagenesis Kit® (Tyobo Co., Osaka, Japan) to construct full-length mutant cDNA p.Ala225Glu. Wild-type and mutant constructs were transfected via Lipofectamine® 2000 (Invitrogen, San Diego, CA, USA) into 5×10^5 SV40-transformed T2-deficient fibroblasts. After incubation at 37 °C for 72 h, cells were harvested and stored at −80 °C until needed. After that, cells were freeze–thawed and sonicated in 50 mM sodium phosphate (pH 8.0) and 0.1% Triton X-100. This was followed by centrifugation at 10,000*g* for 10 min. Supernatants from cell extracts were used for enzyme assay and immunoblot analysis.

We spectrophotometrically monitored the decrease of acetoacetyl-CoA absorbance at 303 nm, which is caused by acetoacetyl-CoA thiolysis to acetyl-CoA. Acetoacetyl-CoA thiolase activity, was measured in the absence and presence of potassium ions. Potassium ions specifically stimulate T2; hence, the difference represents T2 activity [19,21]. We calculated the average and standard errors of three independent experiments. An immunoblot analysis was performed as previously described [19,22]. We used a mixture of an anti-T2 and anti-succinyl-CoA:3-oxoacid CoA transferase (SCOT) antibodies as the first antibody. Serial dilution samples extracted from the wild-type (10, 5, 2.5, and 1.25 μg) were

electrophoresed together with mock and mutant samples (10 μg). Therefore, we could estimate the amount of any detected mutant T2 protein compared to wild-type.

3. Results

Patient 2 has a novel homozygous mutation c.674C > A (p.Ala225Glu) in exon 7 of *ACAT1* gene. This was confirmed by familial analysis; both parents and the younger brother are heterozygotes for the same mutation (Supplementary material 1). The expression of wild-type T2 cDNA yielded high potassium ion-activated acetoacetyl-CoA thiolase activity, which represents T2 activity. In contrast, p.Ala225Glu mutation, like mock cDNA, did not produce any detectable T2 enzyme activity (Fig. 1A). In immunoblot analysis (Fig. 1B), no p.Ala225Glu mutant T2 protein was detected. The smallest used amount of wild type (1.25 μg) was clearly visible. Therefore, the amount of p.Ala225Glu mutant T2 protein, if any, would be 12.5% less than that of wild-type.

4. Discussion

The classically-termed "Arab world" consists of 22 Arabic-speaking countries over approximately 13×10^6 km^2, and has a population of approximately 425 million. Several patients with T2 deficiency have been identified in different Arab countries, mostly through case reports, retrospective studies, or selective screening (Table 1). Emerging nationwide newborn screening (NBS) programs by MS/MS have recently been launched in only a few Arab countries (including Qatar, Saudi Arabia, and the United Arab Emirates) [16]. The incidence of T2 deficiency has been estimated by three Arab studies to be 1.4, 1.6, and 2.76 per 100,000 newborns [11,16,18]. These numbers are higher than the reported figures of 1 per 313,000 in North Carolina, and 1 per 232,000 newborns in Minnesota, USA [23,24]. As some patients may not be detected by NBS programs using MS/MS, the true incidence of T2 deficiency is likely higher [3]. This may be expected due to the high rate of consanguineous marriages among Arab populations [16,18].

The typical presentation of T2 deficiency consists of intermittent episodes of ketoacidosis that are usually triggered by infections, fasting, or increased protein intake. Patients commonly have clinical manifestations seen in metabolic encephalopathies, such as vomiting, convulsions, abnormal tone, and disturbed conscious level. Most patients with T2 deficiency recover uneventfully, as in patient 2 in this paper; however, death or irreversible neurological damage may ensue, as in patient 1 [1,25,26]. Given the unspecific clinical presentation, T2 deficiency may be initially misdiagnosed by primary care physicians. T2 deficiency may mimic central nervous system infection, like in the second episode of patient 1; diabetic ketoacidosis, if associated with stress hyperglycemia; or even poisoning, such as of salicylates. Diagnostic clues towards T2 deficiency include the following: first, a severe ketoacidosis outweighing the associated illness; second, a ratio of free fatty acids to total ketone body (TKB) (both in mmol/L) less than 0.3 during early fasting stages; and third, a significantly high TKB relative to blood glucose (TKB × blood glucose > 14; both in mmol/L) during hypoglycemic states [2,3,27]. Between episodes, patients with T2 deficiency are usually asymptomatic, except for potentially irreversible consequences from a prior severe ketoacidotic episode [1]. Intriguingly, some T2-deficient patients, including 4 Arabs, have been found to have neurodevelopmental impairments without identifiable ketoacidotic episodes [6,28]. Metabolic stroke is increasingly recognized in T2 deficiency not only after acute acidoses, as in mitochondrial respiratory chain and organic acidurias, but also in the absence of frank metabolic decompensation [25,26,28]. Direct neurotoxic effects of accumulated isoleucine catabolic intermediates have been proposed [28,29].

The characteristic abnormalities in blood acylcarnitine (elevated C5:1 and C5-OH), as seen in our patients, and urinary organic acids (increased 2M3HB, 2MAA, and TIG) profiles, usually provide supportive evidence for T2 deficiency. However, some patients whose mutant T2 enzymes retain some residual activities may show subtle or atypical biochemical abnormalities [21]. Moreover, another disorder, 2-methyl-3-hydroxybutyryl-CoA dehydrogenase deficiency, may mimic, in part, the biochemical profile of T2 deficiency [3]. Therefore, a T2 enzyme assay or *ACAT1* mutational analysis is required to confirm the diagnosis. If a novel variant is discovered, like c.674C > A in patient 2, an expression analysis is required to prove its pathogenicity. Accordingly, the diagnosis can be confirmed in the index patient, screening of other family members for yet asymptomatic patients becomes applicable, and

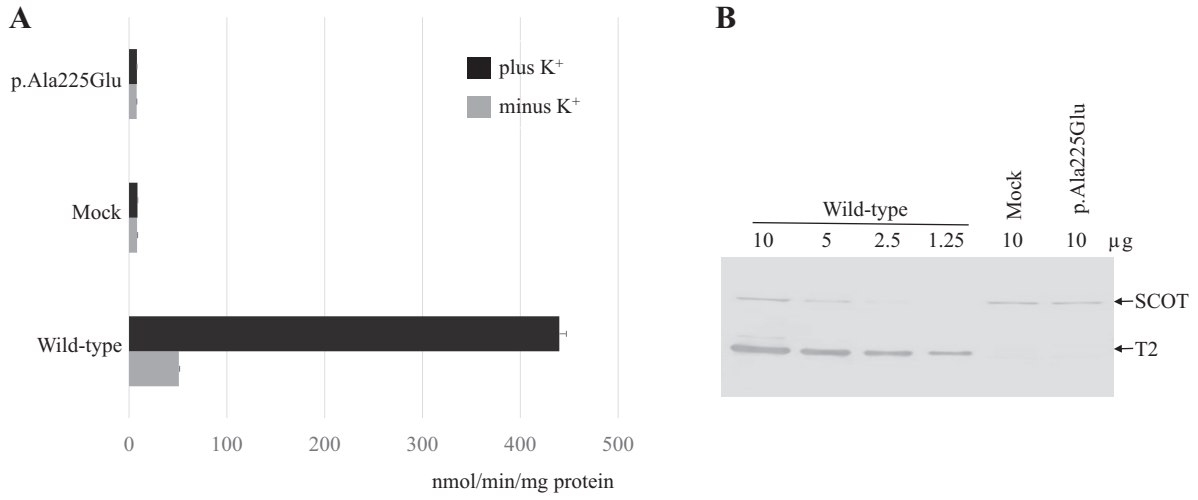

Fig. 1. Transient expression analysis. Wild-type, mock, and p.Ala225Glu mitochondrial acetoacetyl-CoA thiolase (T2) cDNAs were transfected into SV40-transformed T2-deficient fibroblasts. Cells were incubated at 37 °C for 72 h followed by harvesting. After processing, supernatants from cell extracts were used for an enzyme assay and immunoblot analysis. (A) Acetoacetyl-CoA thiolase enzyme assay. The mean and standard errors of three independent experiments, before and after addition of potassium ions, are shown. (B) Immunoblot analysis. Applied protein amounts are demonstrated (in μg) above the lanes. The 1st antibody was a mixture of an anti-T2 and anti-succinyl-CoA:3-oxoacid CoA transferase (SCOT) antibodies. SCOT, succinyl-CoA:3-oxoacid CoA transferase; T2, mitochondrial acetoacetyl-CoA thiolase.

Table 1
Arab patients with beta-ketothiolase deficiency.[a]

Population	Pt no.	Estimated prevalence[b]	Study type	Enzyme assay	*ACAT1* mutation analysis	Ref.
Tunisia	10	–	Case reports	[c]Yes (1)	–	[8]
Egypt	8	2.76	NBS pilot study (1) selective screening (7)	–	–	[9,12,13,18]
Syria	5	–	Retrospective	–	–	[14]
United Arab Emirates	3	1.4	NBS program (1) retrospective (2)	–	[e]c.86_87dupTG, c.854C > T	[11,15]
Saudi Arabia	3	–	Case report	[c]Yes	–	[6]
Iraq	3	–	Selective screening	–	–	[17]
Oman	2	–	Selective screening	–	–	[10]
Lebanon	2	1.6	Limited NBS program	–	–	[16]
Libya	2	–	Case report	–	c.674C > A	Current study
Yemen	1	–	Case report	–	–	[6]
Palestine	1	–	Case report	[d]Yes	–	[7]

[a] This may not be an exhaustive list.
[b] Per 1×10^5 newborns.
[c] Potassium ion-activated acetoacetyl-CoA thiolase assay.
[d] Coupled assay for detection of defects in isoleucine catabolism distal to enoyl-CoA hydratase, using tiglyl-CoA as a substrate.
[e] Pathogenicity of these variants have not been confirmed to date. NBS, newborn screening; Pt, patient.

proper genetic counseling can be provided [3]. A prenatal diagnosis is also possible. However, it is not necessarily superior to a timely postnatal diagnosis because T2 deficiency is rarely manifested during the neonatal period [1]. Of note, T2 deficiency shows no correlation between genotype and clinical phenotype [1,30].

The management of acute ketoacidotic episodes in T2 deficiency is aimed at stabilizing the patient's condition and correcting metabolic derangements. Intravenous glucose is started to suppress ketogenesis; blood glucose should be maintained at a high-normal range. Appropriate intravenous fluids and electrolytes are provided; bicarbonates are only used in severe metabolic acidosis. Other supportive measures, such as mechanical ventilation, may also be required. All measures are guided by frequent monitoring of the patient's clinical and biochemical conditions [1–3]. The mainstay of precautionary management for T2 deficiency includes avoidance of prolonged fasting and early management of infections. Intravenous glucose should be administered when an associated illness precludes oral intake. Other non-evidence-based measures, such as mild protein restriction, avoidance of excessive fat intake, and carnitine supplementation, are also commonly advised [1,3]. As patient 2 manifested after his younger sister (patient 1) had already been diagnosed with T2 deficiency, a timely diagnosis, and both acute and long-term management, were assured. This likely contributed to the favorable outcome in patient 2.

In conclusion, we report on two siblings with T2 deficiency from Libya. Both siblings were presented with ketoacidosis, but the severity and outcomes were quite distinctive. We identified a novel *ACAT1* mutation (c.674C > A) and proved its pathogenicity by transient expression analysis. The incidence of T2 deficiency may be high among the Arab population. This disease should be considered in the differential diagnosis of unexplained ketoacidosis and in certain clinical circumstances. Patients with T2 deficiency could have more favorable outcomes with early diagnosis and proper management.

Contributions of individual authors

E. Abdelkreem and Y. Aoyama collected data, performed mutational and expression analyses, and drafted the first version of manuscript. H. Alobaidy was involved in clinical management of patients and critically reviewed the manuscript. S. Mahmoud and M. Abd El Aal critically reviewed and revised the manuscript. T. Fukao supervised the study and reviewed and revised the manuscript. All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work. All authors confirm the absence of previous similar or simultaneous publications.

Conflict of interest

The authors declare no potential conflicts of interest on the research, authorship, and publication of this article.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejmhg.2016.11.001.

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