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RESEARCH REPORT

Clinical and Mutational Characterizations of Ten Indian Patients with Beta-Ketothiolase Deficiency

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Abstract Beta-ketothiolase deficiency (mitochondrial acetoacetyl-CoA thiolase (T2) deficiency) is an inherited disease of isoleucine catabolism and ketone body utilization caused by *ACAT1* mutations. We identified ten Indian patients who manifested with ketoacidotic episodes of variable severity. The patients showed increased urinary excretion of isoleucine-catabolic intermediates: 2-methyl-3-hydroxybutyrate, 2-methylacetoacetate, and tiglylglycine. Six patients had a favorable outcome, one died, and three developed neurodevelopmental sequela. Mutational analysis revealed a common (p.Met193Arg) and four novel (p.Ile323Thr, p.Ala215Asn, c.1012_1015dup, and c.730+1G>A) *ACAT1* mutations. Transient expression analyses of wild-type and mutant cDNA were performed at 30, 37, and 40°C. A p.Ile323Thr mutant T2 was detected with relative enzyme activity and protein amount of 20% and 25%, respectively, compared with wild type at 37°C; it was more prevalent at 30°C but ablated at 40°C. These findings showed that p.Ile323Thr had a significant residual T2 activity with temperature-sensitive instability. Neither residual enzymatic activity nor mutant T2 protein was identified in p.Met193Arg, p.Ala215Asn, and c.1012_1015dup mutations using supernatants; however, these mutant T2 proteins were detected in insoluble pellets by immunoblot analysis. Expression analyses confirmed pathogenicity of these mutations. T2 deficiency has a likely high incidence in India and p.Met193Arg may be a common mutation in the Indian population.

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Abbreviations

2MAA	2-Methylacetoacetate
2M3HB	2-Methyl-3-hydroxybutyrate
SCOT	Succinyl-CoA:3-oxoacid CoA transferase
TIG	Tiglylglycine
T2	Mitochondrial acetoacetyl-CoA thiolase

Introduction

Beta-ketothiolase deficiency, also referred to as mitochondrial acetoacetyl-CoA thiolase (T2) (EC 2.3.1.9, gene symbol *ACAT1*) deficiency (Online Mendelian Inheritance in Man [OMIM] 203750, 607809), is an autosomal recessive disease of isoleucine catabolism and ketone body utilization (Fukao et al. 2014; Hori et al. 2015). Since the first description of T2 deficiency in 1971, more than 100

patients with the condition have been identified worldwide with no ethnic predisposition (Abdelkreem et al. 2016). The clinical hallmark of this disease is recurrent ketoacidotic episodes, between which patients usually have no symptoms. T2 deficiency is marked with increased urinary excretion of the isoleucine catabolic intermediates 2-methyl-3-hydroxybutyrate (2M3HB), 2-methylacetoacetate (2MAA), and tiglylglycine (TIG). The clinical severity of T2 deficiency varies among patients, with some reports of atypical clinical/biochemical presentations. Information extrapolated from follow-up studies of patients with T2 deficiency suggests that a favorable outcome is usually anticipated unless a ketoacidotic episode gives rise to fatal or irreversible, predominantly neurological, complications (Fukao et al. 2014; Abdelkreem et al. 2016).

The human *ACAT1* gene is located at chromosome 11q22.3–23.1. It spans approximately 27 kb and contains 12 exons and 11 introns. T2 cDNA is about 1.5 kb long and encodes a precursor protein of 427 amino acids, including a 33-amino-acid leader polypeptide (Fukao et al. 1990). Mutations in the *ACAT1* gene are highly heterogeneous; to date, more than 70 mutations have been identified (Fukao et al. 2010; unpublished data). Apart from the p. Arg208* mutation that was detected in Vietnamese patients with T2 deficiency, no other common *ACAT1* mutations were identified before this study (Abdelkreem et al. 2016).

There are few studies on T2 deficiency in India. Some cases were detected by urinary organic acid analysis, but the diagnosis was not confirmed by an enzyme assay or mutational analysis except for only one case (Dave Usha and Das Bibhu 2010; Akella et al. 2014). In this study, we report on ten Indians with T2 deficiency, describe their clinical and molecular features, and characterize four *ACAT1* mutations.

Patients and Methods

Patients

The ten Indian patients, who were not related to each other, presented with ketoacidotic episodes of variable severity (Table 1). Most were from Hyderabad and were of Hindu heritage. Suspicion of T2 deficiency in the patients was based on increased urinary excretion of 2M3HB, 2MAA, and TIG, and increased C5:1 carnitine and C5-OH carnitine in the blood acylcarnitine profile; data of urinary organic acid profiles were available for all patients, whereas that of blood acylcarnitine were available for only six patients. Among the ten patients, one patient, GK108, died during his presenting ketoacidotic episode at 11 months of age; GK98 experienced only one ketoacidotic episode at 4 months of age, which has been complicated with severe neurodevelopmental regression and convulsions; GK109 and GK113 both suffered modest developmental delays after their second ketoacidotic episodes; and the others, in contrast, have achieved age-appropriate development to date.

Ethical Considerations

This study was approved by the Ethical Committee of the Graduate School of Medicine, Gifu University, Japan, and was carried out in accordance with the principles contained within the Declaration of Helsinki. Informed consents were obtained from all patients or their parents for being included in the study.

Mutation Analysis

Genomic DNA was extracted and purified from patients' blood using a SepaGene kit (EIDIA, Tokyo, Japan) according to the manufacturer's directions. The 12 *ACAT1* exons, with their intron boundaries, were amplified by PCR using formerly designed primer pairs and conditions (Fukao et al. 1998). The 12 amplified fragments were sequenced using a BigDye® Terminator v1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and an ABI PRISM 3130XL genetic analyzer (Applied Biosystems) according to the manufacturer's directions. We used the genomic *ACAT1* sequence (GenBank accession NG_009888.1) as a reference.

Transient Expression Analyses

Transient expression analyses of T2 cDNA were performed using a pCAGGS eukaryote expression vector, as previously described (Niwa et al. 1991; Fukao et al. 1998; Zhang et al. 2004). Briefly, we used a Kod-Plus-Mutagenesis Kit® (Tyobo Co., Osaka, Japan) to construct four full-length mutant cDNA: p.Met193Arg, p.Ile323Thr, p.Ala215Asn, and c.1012_1015dup. Wild-type and mutant constructs were transfected via Lipofectamine2000® (Invitrogen, San Diego, CA, USA) into 5×10^5 SV40-transformed T2-deficient fibroblasts and cultured at 37°C. After 24 h, cells were incubated at 30, 37, and 40°C for another 48 h. Thereafter, cells were harvested and kept at −80°C until use. Cells were freeze-thawed and sonicated in 50 mM sodium phosphate (pH 8.0) and 0.1% Triton X-100 followed by centrifugation at 10,000×g for 10 min.

Using supernatants from cell extracts, we spectrophotometrically monitored the decrease of acetoacetyl-CoA absorbance at 303 nm, which is the result of thiolysis of acetoacetyl-CoA to acetyl-CoA. We measured the difference of thiolase activity in the absence and the presence of potassium ions, which specifically stimulate T2; such a difference represents T2 activity (Fukao et al. 1998; Zhang et al. 2004). The average of three independent experiments

Table 1 Clinical, biochemical, and molecular summary of Indian patients with beta-ketothiolase deficiency

			The first crisis											Prognosis			Mutations	
ID	Consanguinity	Sex	Age at onset	Preceding illness	pH	HCO_3	BE	Glucose	NH_3	Ketonuria	Unconsciousness (No. of days)	Mechanical ventilation	Peritoneal dialysis	No. of crises	Present age	Development outcome		
GK94	+	M	10 months	Fever, cough	6.89	3.3	−28	1.4	22	>80 mg/dl	Coma (4)			1	5.5 year	Good	c.253_255del (p.Glu85del)	c.253_255del (p.Glu85del)
GK98	–	M	4 months		6.8		−30	4.5	37	++++	Coma (7)	+	+	1	3.5 year	Impaired	c.578T>G (p.Met193Arg)	c.578T>G (p.Met193Arg)
GK99	–	M	14 months	Fever, cough	6.9	3.2	−28	1.8		160 mg/dl	Coma (2)	+	+	1	3 year	Good	c.578T>G (p.Met193Arg)	c.578T>G (p.Met193Arg)
GK108	+	F	11 months	Vomiting	6.9	7.2	−22.5				Coma (1)	+		1	Died at 11 month		c.578T>G (p.Met193Arg)	c.578T>G (p.Met193Arg)
GK109	–	M	9 months	Cough	6.8	1.9	−30.5	12.3	116	++++	Coma (1)	+	+	2	2 year	Impaired	c.1012_1015dup	c.1012_1015dup
GK110	+	F	11 months		7	2.4	−28	14.7	42	160 mg/dl	Coma (3)			1	2 year	Good	c.730+1G>A	c.730+1G>A
GK111	–	M	6 months	Fever, cough	7.1	2.4		3.8	49	>80 mg/dl	Coma (4)	+		1	1.5 year	Good	c.578T>G (p.Met193Arg)	c.968T>C (p.Ile323Thr)
GK112	+	F	7 months	Fever, cough	7.15	20	−8		50	+++	lethargy (3)	+		3	2.5 year	Good	c.1124A>G	c.1124A>G
GK113	+	M	12 months	Fever, cough	7.1						Coma (6)			2	2.5 year	Impaired	c.643_644delinsAA (p.Ala215Asn)	c.643_644delinsAA (p.Ala215Asn)
GK114	–	M	19 months	Fever, cough	6.9	3				50 mg/dl	Coma (1)	+	+	1	3 year	Good	c.578T>G (p.Met193Arg)	c.578T>G (p.Met193Arg)

Values for pH, HCO_3, BE, and glucose are shown in mmol/L, whereas NH_3 is in µmol/L. These patients were diagnosed between 2011 and 2016. All patients were from Hyderabad except GK109 and GK112, who were from Mumbai. Patients' development was assessed using the Denver II test (http://denverii.com)

was calculated. In addition, both supernatants and pellets of cell extracts were subjected to immunoblot analysis as described (Fukao et al. 1997, 1998). The first antibody was a mixture of an anti-T2 antibody and anti-succinyl-CoA:3-oxoacid CoA transferase (SCOT) antibody, as previously described (Fukao et al. 1997).

Results

Mutation Analysis

We identified four novel *ACAT1* mutations: c.968T>C (p.Ile323Thr), c.643_644delinsAA (p.Ala215Asn), c.1012_1015dup, and c.730+1G>A. c.578T>G (p.Met193Arg) was the most common mutation, representing 45% of all identified mutant alleles (Table 1).

Transient Expression Analysis

As shown in Fig. 1a, expression of wild-type T2 cDNA produced high-potassium ion-activated acetoacetyl-CoA thiolase activity, which represents T2 activity, whereas that of mock cDNA yielded no detectable T2 enzyme activity at various temperatures. The p.Ile323Thr mutation retained some residual T2 activity, nearly 20%, compared with the wild type, through expression at 37°C. At 30°C, such activity increased to 40%, whereas it was completely ablated at 40°C. Conversely, p.Met193Arg, p.Ala215Asn, and c.1012_1015dup mutations showed no detectable T2 activity through expression under the different temperatures.

In immunoblot analysis of supernatants (Fig. 1b), serial dilution samples extracted from the wild type were applied to evaluate relative amounts of mutant T2 proteins in comparison with that of the wild type. p.Ile323Thr mutant T2 protein was detected through expression at 30 and 37°C but not 40°C. Relative amounts of p.Ile323Thr mutant T2 protein, compared with the wild type, were estimated to be 40% and 20% at 30°C and 37°C, respectively. p.Met193Arg, p.Ala215Asn, and c.1012_1015dup mutant T2 proteins were not observed using supernatants through expression at the designed temperatures; however, these mutant proteins were clearly identified using pellet fractions, with notably faster electrophoretic mobility of the latter mutant protein (Fig. 1c).

Estimated Incidence

The ten Indian patients with T2 deficiency manifested with ketoacidotic episodes between 2011 and 2016. Eight patients were from Hyderabad, which is located in Southern India, covers 650 km^2, and has a population of about 10 million. The birth rate in this area is about 200,000 newborns/year. These eight patients and the previously reported T2-deficient case (GK95), who also was from Hyderabad (Akella et al. 2014), were born between 2010 and 2014. Accordingly, if these nine patients represented all T2-deficient patients during these 5 years in Hyderabad, the incidence of T2 deficiency would be about 1 in 111,000 newborns in this area (9 T2-deficient patients/200,000 $\times$ 5 years). This estimated figure is strikingly greater than the highest ever reported incidence of T2 deficiency, which is 1 per 232,000 newborns in Minnesota, USA, between January 2001 and November 2010 (Sarafoglou et al. 2011). As it is possible that other T2-deficient patients died or suffered mild episodes without being diagnosed (Abdelkreem et al. 2016), the actual incidence of T2 deficiency in Hyderabad from 2010 to 2014 may be even higher.

Discussion

ACAT1 mutations are highly diverse, with more than 70 different mutations having been identified to date, and with only a few common mutations (Fukao et al. 2010; Hori et al. 2015). p.Met193Arg accounts for about half the identified mutant alleles in Indian patients with T2 deficiency, suggesting a founder effect in this Indian region. p.Met193Arg is the second most common *ACAT1* mutation after p.Arg208*; the latter mutation represents 87% of T2 mutant alleles in Vietnamese (Fukao et al. 2010). Patients homozygous for p.Met193Arg have, in general, typical clinical and biochemical manifestations of T2 deficiency, although clinical severity and outcome vary. Potentially common *ACAT1* mutations that have yet to be identified could be revealed through the ongoing recognition of more patients with T2 deficiency, particularly in populations with a high rate of consanguineous marriage.

Among the four novel *ACAT1* mutations identified in the current study, c.730+1G>A is an apparent disease-causing mutation because it affects a highly conserved point at the splice donor site of intron 7; aberrant splicing usually ensues as a consequence of decreasing the Shapiro and Senapathy score by c.730+1G>A mutation from 72 to 55 at that splice site (Shapiro and Senapathy 1987). We performed transient expression analysis to confirm that the two novel missense mutations, p.Ile323Thr and p.Ala215Asn, are pathogenic. p.Met193Arg mutation was included as its enzyme activity was analyzed by expression at only 37°C (Akella et al. 2014). c.1012_1015dup is another plausibly detrimental mutation. It causes a frame shift that replaces aspartic acid at position 339 with glutamic acid and inserts a premature stop codon at position 355 of the new reading frame (p.Asp339GlufsTer17). We also studied this mutation

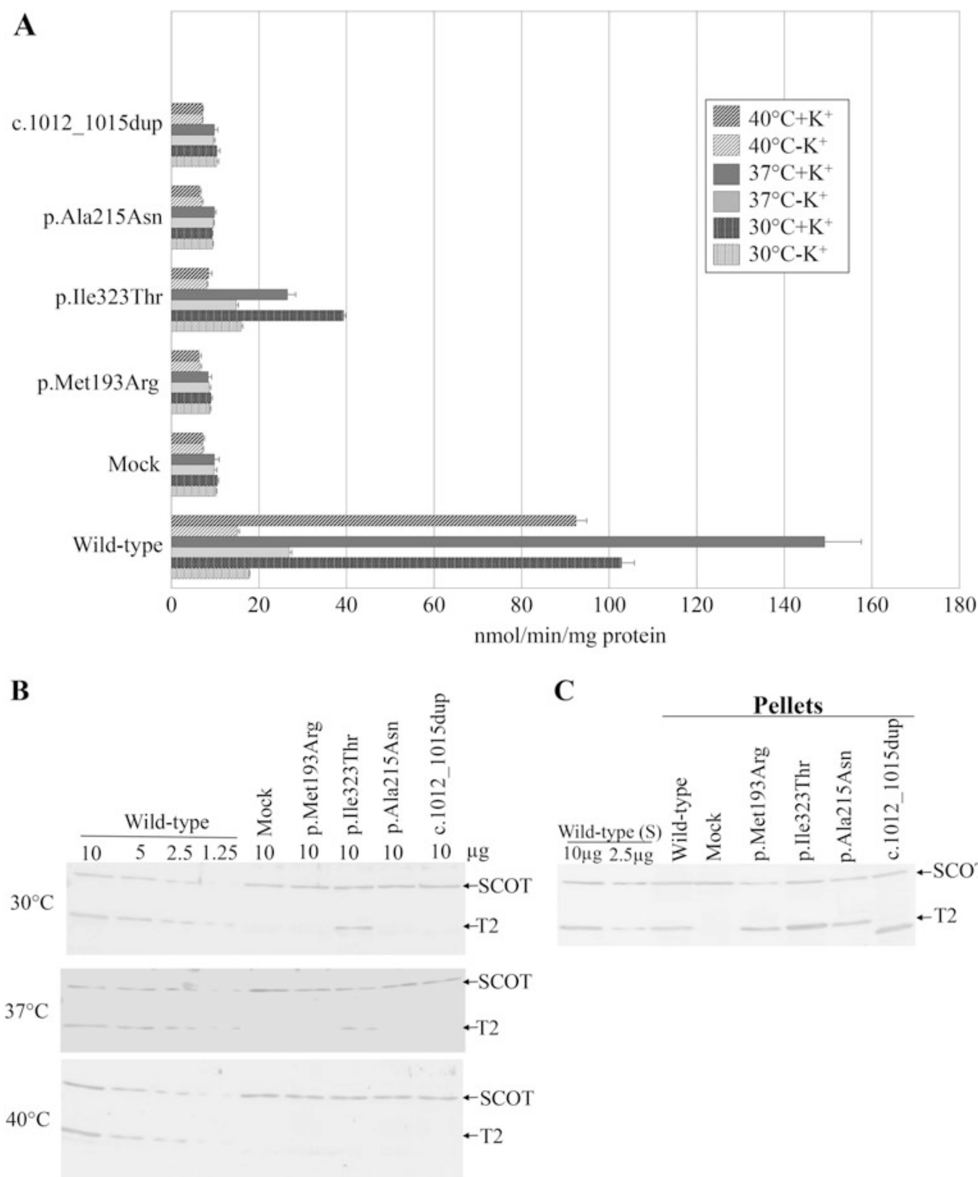

Fig. 1 Transient expression analyses of mutant cDNA. Wild-type and mutant constructs were transfected using Lipofectamine2000® into 5×10^5 SV40-transformed T2-deficient fibroblasts. After incubation at 37°C for 24 h, cells were incubated at 30, 37, and 40°C for 48 h. (**a**) Potassium ion-activated acetoacetyl-CoA thiolase assay. Mean values of acetoacetyl-CoA thiolase activity measured in supernatants of cell extracts in the absence and the presence of potassium ions are shown together with the standard errors of three independent experiments. (**b**) Immunoblot analyses of supernatants. A mixture of an anti-T2 and anti-SCOT was used as the first antibody. Applied protein amounts are shown (in μg) above the lanes. (**c**) Immunoblot analyses of pellets. S indicates supernatant of the wild type that was used in amounts shown above the lanes. Pellets from samples corresponding to 10 μg of supernatant protein were applied. *SCOT* succinyl-CoA:3-oxoacid CoA transferase, *T2* mitochondrial acetoacetyl-CoA thiolase

to elucidate the electrophoretic characters of its mutant protein. The pathogenic effect of the p.Glu85del mutation on T2 enzyme activity was previously demonstrated (Fukao et al. 2002), whereas c.1124A>G was shown to induce a cryptic splice donor site within exon 11 that results in aberrant splicing (Fukao et al. 2008).

The results of T2 enzyme assay and immunoblot analysis clearly showed that p.Ile323Thr mutant T2 protein has a significant residual T2 activity and is unstable in a temperature-sensitive manner, being more prevalent by expression at lower temperatures (30°C > 37°C > 40°C). The other mutant proteins, p.Met193Arg, p.Ala215Asn, and c.1012_1015dup, were not detected in supernatant fractions even at 30°C, suggesting that they are very unstable mutant proteins. The incubation temperature affects posttranslational modifications of protein structure, including protein folding and stability. Incubation at a lower temperature during expression may considerably stabilize mutant T2

proteins (Sakurai et al. 2007). This temperature-sensitive phenomenon was previously described in several *ACAT1* mutations, such as p.Ala132Gly, p.Gln145Glu, p.Glu215-del, p.Glu252del, and p.Thr297Met (Fukao et al. 2001; Zhang et al. 2004; Sakurai et al. 2007).

To further study the effects of mutations on protein solubility, we examined not only supernatants used for the enzyme assay but also pellets of cell extracts. Insoluble mutant T2 proteins p.Ala215Asn, p.Met193Arg, and c.1012_1015dup were detected only in pellets. These three mutations appear to have drastic effects on T2 protein structure, like p.Ala333Pro, which was also identified only in pellets (Fukao et al. 1998). Although having no residual enzymatic activities, some mutant T2 proteins are soluble. For example, p.Gly152Ala and p.Asn158Asp were detected by expression at 30°C, whereas others, such as p. Arg208Gln, p.Tyr219His, and p.Asn282His, could be identified in supernatants by expression not only at 37°C but also at 40°C (Zhang et al. 2004; Sakurai et al. 2007).

A distinction between genetic mutations based on their residual enzymatic activities may become important for some prospective therapeutic modalities. Molecular chaperones, polypeptide unfoldases, can specifically recognize and proofread three-dimensional structures of misfolded/aggregated proteins and convert them into degradable or rehabilitated, potentially functional, proteins. Stimulation of the latter mechanism using various pharmacological agents may favorably affect functions of misfolded mutant proteins if they have residual enzymatic activities, offering potential hope for a variety of yet untreatable genetic diseases (West et al. 2012; Muntau et al. 2014; Finka et al. 2016). The clinical course of T2-deficient patients whose mutations have some residual enzyme activity, such as GK111 here, does not differ from those whose mutations have no enzymatic activity (Fukao et al. 2001). Such lack of genotype-phenotype correlation precludes the use of unfoldases as a potential therapy for T2 deficiency.

In conclusion, we confirmed T2 deficiency at the molecular level in ten new patients from India and characterized their *ACAT1* mutations. T2 deficiency has a likely high incidence in India and p.Met193Arg may be a common mutation in the Indian population.

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Synopsis

Beta-ketothiolase (T2) deficiency has a likely high incidence in India where p.Met193Arg may be a common mutation.

Compliance with Ethics Guidelines

Conflict of Interest

Toshiyuki Fukao has received a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan [grant numbers 26114708, 24591505, 16K09962]; Health and Labour Science Research Grants for Research on Intractable Diseases from the Ministry of Health, Labour and Welfare of Japan; and the Practical Research Project for Rare/Intractable Diseases from Japan Agency for Medical Research and Development (AMED).

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Informed Consent

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 patients or their parents for being included in the study.

Details of the Contributions of Individual Authors

Elsayed Abdelkreem, Hiroki Otsuka, Hideo Sasai, Yuka Aoyama, and Mina Nakama collected data, performed mutational and expression analyses, and drafted the first version of the manuscript. Radha Rama Devi Akella, Usha Dave, and Sudhir Sane were involved in clinical management of patients and critically reviewed the manuscript. Hidenori Ohnishi, Shaimaa Mahmoud, and Mohamed Abd El Aal critically reviewed and revised the manuscript, and approved the final version as submitted. Toshiyuki Fukao

initiated and supervised this study, reviewed and revised the manuscript, and approved the final version as submitted. 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.

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