ARGININOSUCCINATE SYNTHETASE DEFICIENCY: MUTATION ANALYSIS IN 3 THAI PATIENTS

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Abstract. Remarkable improvements in public health, nutrition, hygiene, and availability of medical services in the last 20 years have significantly reduced infant and childhood mortality in Thailand. Therefore, many rare and previously unidentified genetic disorders, which, in the past, usually led to the death of affected infants before a definitive diagnosis, have now been increasingly recognized. Recently, we identified three unrelated patients from Thailand who suffered from citrullinemia, one of five inherited types of urea cycle disorders. All were diagnosed within their first few weeks of life. Biochemical analyses, including plasma amino acid and urine organic acid profiles, are consistent with argininosuccinate synthetase (*ASS*) deficiency. Extensive mutation study by direct genomic sequencing of *ASS* demonstrated a homozygous G117S mutation in one patient and homozygous R363W mutations in the other two families.

INTRODUCTION

Disorders of the urea cycle, a major component of amino acid metabolism in the liver, result from deficiencies of several key, rate-limiting enzymes in the cascade utilizing ammonia and commonly characterized by hyperammonemia. Five inherited disorders of the urea cycle, due to different enzymatic defects, have been well described (Brusilow and Horwich, 2001). Classical citrullinemia (Type I; MIM 215700) is one of these inborn errors of urea cycle metabolism, resulting in a marked elevation of plasma citrulline and ammonia, and caused by a deficiency of argininosuccinate synthetase (*ASS*) (EC 6.3.4.5), an enzyme which converts citrulline to argininosuccinate. It is widely observed that the clinical presentation in patients with citrullinemia is highly variable: from early onset in neonatal or late infancy to late onset in puberty (Leonard, 2000). In the early onset condition, affected newborns present with severe lethargy, poor feeding to respiratory distress, jitteriness and neonatal seizures. Clinical presentations are usually indistinguishable from neonatal infection/sepsis. Without measurements of plasma ammonia, plasma amino acids, urinary organic acids and orotic acid to provide direct evidence for urea cycle disorders, a definitive diagnosis of citrullinemia is a difficult task in developing countries, where infection/sepsis remains the major cause of death during the neonatal and infancy period. Diagnostic services for inborn errors of metabolism in Thailand were established at Siriraj Hospital, Bangkok in 2001 and more than 150 patients with inherited metabolic disorders have been definitively diagnosed. Here, we report three patients with citrullinemia, who have been extensively analyzed at the biochemical and molecular level. Two recurrent argininosuccinate synthetase mutations have been identified and suggest at least one mutation hotspot due to deamination of 5′ methylcytosine and a subsequent repair defect of base-mispairing.

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PATIENTS AND METHODS

Patients

Patient 1. A 7-week-old female infant was born in September 1999 at term, after uneventful pregnancy. She was the first child of a Pakistani couple with a history of consanguinity. She developed recurrent vomiting beginning on day 6 after birth and was diagnosed as having neonatal sepsis and pyloric stenosis. After two months of medical management in a neonatal intensive care unit, including surgical correction for pyloric stenosis, she was discharged from a private hospital. Her condition deteriorated, and she subsequently developed hyperammonemic coma on day 53, when she was referred to our hospital. Quantitative plasma amino acid analysis demonstrated marked elevation of citrulline (2,960 nmol/ml, normal 0.00-108.08 nmol/ml) and glutamine 1,590 nmol/ml (normal 52.39-727.67 nmol/ml). The patient received specific management, including double blood volume exchange-transfusions (x 2), L-arginine and sodium benzoate substitution and a low protein diet. The clinical condition markedly improved and she was discharged after two weeks. The patient is now 4 years old and attending a special school for children with disability; walks with aid, speaks 2-word-sentences and has moderately delayed development. (Wasant *et al* 1999, 2002 a,b).

Patient 2. A 28-day-old male infant born in May, 2002 was referred to our hospital in shock and comatose condition (day 29). The patient developed respiratory distress and lethargy beginning on day 7 after birth, was diagnosed with neonatal sepsis and received intravenous fluid resuscitation and appropriate antibiotics at a provincial hospital. Despite appropriate management for neonatal infection for two weeks, the patient developed intractable seizures, had a deterioration of consciousness into a deep coma; developed subsequent apnea and cardiorespiratory arrest. The plasma citrulline level was 809.69 nmol/ml and the glutamine level was 2,389.52 nmol/ml on admission, and increased to 3,025.22 nmol/ml and 1,249.29 nmol/ml, respectively, 5 days later. He was rescued from hyperammonemic coma (plasma ammonia 609 μmol/l) by supportive and specific treatments including double blood volume exchange transfusions (x 5), L-arginine substitution, Urea Cycle Disorder (UCD) formula, control of infection and a low-protein diet. A brain imaging study by computerized tomography (CT) at 2 months demonstrated liquefaction necrosis from toxic metabolic encephalopathy. He later developed spasticity, generalized tonic-clonic seizures and eventually died at 6 months of age from superimposed nosocomial infection.

Patient 3. An 18-day-old male infant born in November, 2002 was referred to our hospital in coma. The patient developed recurrent poor feeding, lethargy, and seizures since day 2 after birth, was diagnosed with neonatal sepsis/meningitis and received appropriate management for these conditions. However, his clinical condition deteriorated and he later developed apnea requiring ventilatory support. Quantitative plasma amino acid analysis demonstrated elevation of citrulline (944 nmol/ml) and glutamine (3,506 nmol/ml). Despite adequate and appropriate treatment, including peritoneal dialysis, the computerized brain tomography demonstrated generalized encephalomalacia from metabolic encephalopathy and the patient expired at 8 months of age.

Molecular study of the argininosuccinate synthetase gene

DNA was extracted from the peripheral blood leukocytes of all affected individuals using standard phenol-chloroform extraction. All human materials were collected after informed consent. The entire coding sequences (exons 3-16) and all exon-intron boundaries of the human argininosuccinate synthetase gene (ASS) were amplified by polymerase chain reaction (PCR) using the primers described by Haberle *et al* (2002) with some modifications. The reactions were performed using 100 ng of genomic DNA, 25 pmol of each forward and reverse primer, 1.5 mM $MgCl_2$, 16.6 mM $(NH_4)_2SO_4$, Tris-HCl pH 8.8, 200 μM of each dNTP and 2.5 U of Taq polymerase (Roche Diagnostic, Mannheim, Germany) in a final volume of 50 μl. An annealing temperature of 55ºC was used for most PCRs except one (exon 5 at 52ºC). All amplicons were subsequently sequenced using fluorescently labeled

dideoxyterminators (Perkin Elmer Biosystems, Foster City, USA) and analyzed on a 3100-automated capillary sequencer (Applied Biosystems, USA). The sequencing results were compared with the consensus sequence of the ASS gene in the public domain (Genbank accession number AL354898).

To confirm the two ASS mutations (G117S and R363W) detected by direct genomic sequencing, we employed a restriction endonuclease based analysis. In the G117S mutation, a nucleotide substitution, 349G→A (cDNA sequence), identified in the exon 5 from the family 1 (NP) abolishes a restriction site of *Btg* I. The PCR-products of the exon 5 (372 bp) were digested with *Btg* I for four hours according to the manufacturer's instructions (New England Biolabs, Massachusetts, USA). In the wild-type allele (G), *Btg* I digests the PCR products into 264 and 108 bp-fragments, while in the mutant the PCR fragments remain undigested (Fig 3). In R363W (family 2 and 3), a C→T mutation (nucleotide 1087) abolishes the *Hpa* II site. The PCR amplicons of the wild-type exon 14 were digested resulting in 150 and 114 bp fragments, but the mutant (264 bp) was not (Fig 4). The PCR fragments were electrophoresed on 2.5% agarose-TBE gels and visualized after ethidium bromide staining on a UV transilluminator.

Biochemical stadies

Biochemical studies included plasma amio acids were quantitated by high preformance liquid chromatography (HPLC) (Cohen *et al,* 1987) and urine organic acids were analysed by gas-liquid chromatography and mass spectrometry (GC/MS) (Kuhara *et al,* 1999).

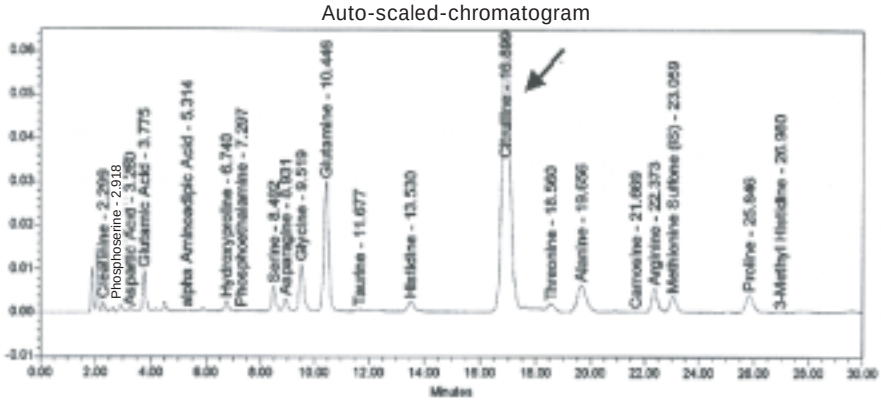

Fig 1–Quantitative plasma amino acid analysis (in patient #2) demonstrating marked elevation of citrulline level.

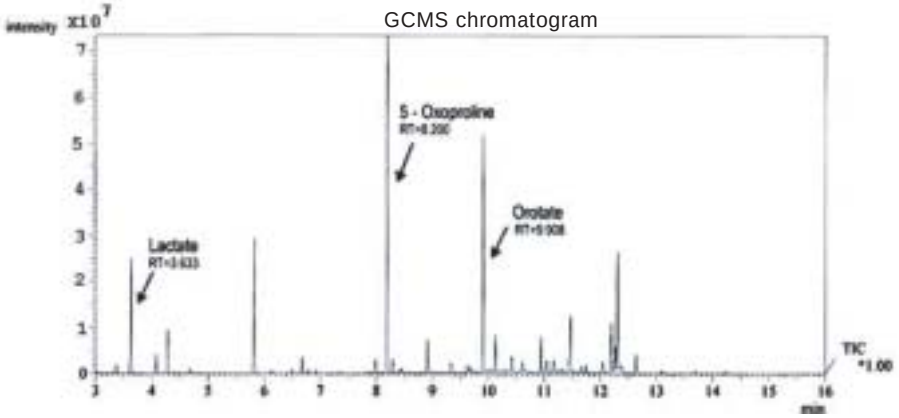

Fig 2–Urine organic acid analysis by gas-liquid chromatography and mass spectrometry (in patient #3) demonstrating orotate.

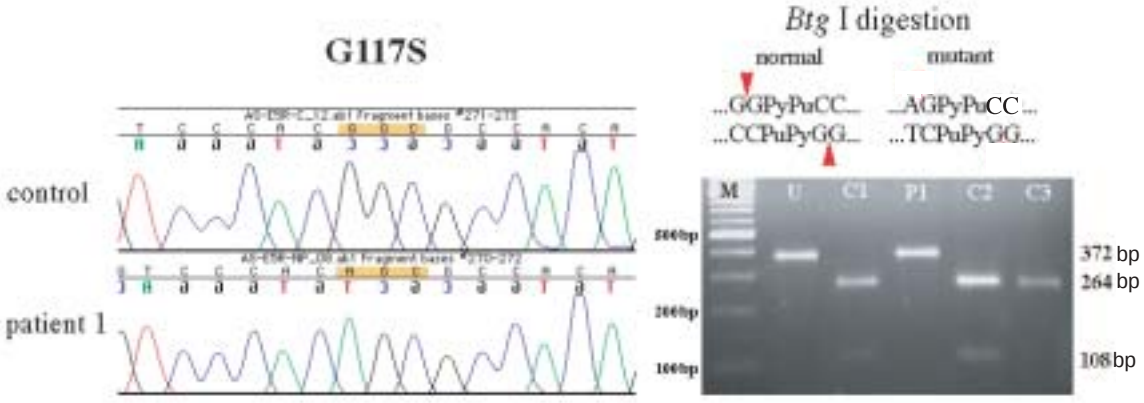

Fig 3–Sequencing analysis of DNA from patient #1 (P1) showing a G→A substitution at nucleotide 349 (G117S)(Left panel) and restriction enzyme analysis by *Btg* I (Right panel). The recognition site (GGPyPuCC) for Btg I is demonstrated and this site is abolished by a substitution of A to G. P1 = patient #1 and C = control sample.

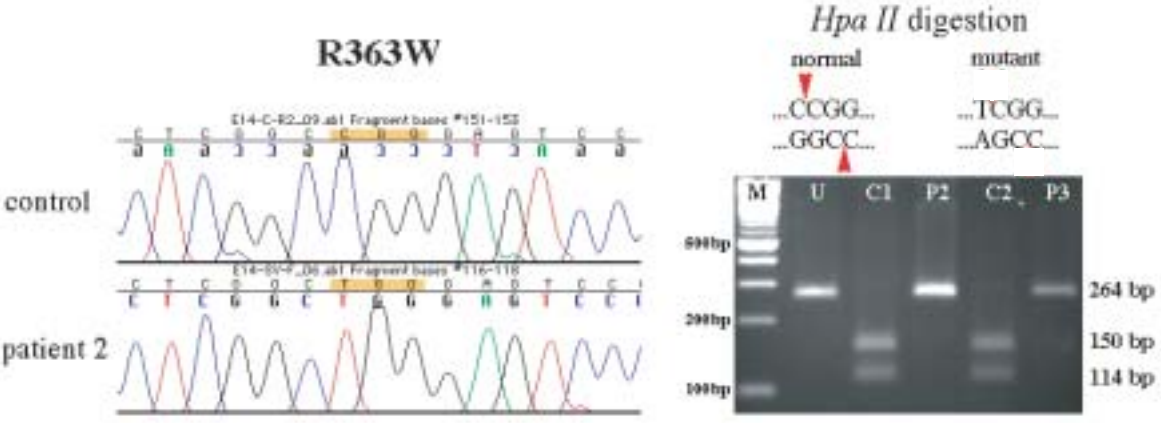

Fig 4–Sequencing analysis of DNA from patient #2 (P2) showing a C→T substitution at nucleotide 1087 (R363W)(Left panel) and a restriction enzyme analysis by *Hpa* II (Right panel). The recognition site (CCGG) for *Hpa* II is shown and this site is abolished by substitution of T to C. P2 = patient #2, P3 = patient #3 and C = control sample. Sequencing results from the patient #3 are not shown.

RESULTS

Quantitative plasma amino acids in patients #2 were presented in Fig 1 and urine organic acids in patient #3 were shows in Fig 2. We were unable to perform enzyme assays in these patients, due to lack of enzyme laboratory facilities in Thailand.

Direct genomic sequencing of the ASS gene successfully identified two different mutations in three unrelated patients from Thailand. The first proband (P1) was found to be homozygous for a codon 117, G→A mutation which substitutes a glycine residue with serine (Fig 3) and this missense mutation was subsequently confirmed by *Btg* I digestion (Fig 3). Patients 2 and 3 (P2 and 3) who were apparently unrelated, were found to be homozygous for the same mutation at codon 363, C→T resulting in tryptophan replacing arginine residue (R363W) (Fig 4). Digestion of the PCR fragments with *Hpa* II confirmed this sequencing result.

DISCUSSION

Neonates who have urea cycle disorders usually progress rapidly from poor feeding, vomiting, lethargy, irritability and tachypnea to seizures, coma and respiratory arrest. The most

important early laboratory test is measurement of the plasma ammonia concentration. Quantitative plasma amino acid analysis and urine organic acid analysis should allow more affected newborns to be diagnosed early and promptly treated to reduce morbidity and mortality (Goodman and Greene, 1994). Since Thai pediatricians, due to their lack of awareness, do not consider inborn errors of metabolism early enough in sick neonates, diagnosis is often delayed, leading to delayed treatment and poor outcome.

We present the first 3 reported cases of citrullinemia in Thailand with extensive biochemical and molecular analysis. The mutation analysis in case #1 is consistent with previous reports in a German patient, while the other 2 Thai patients' mutation was previously reported in a US patient (Gao *et al,* 2003). Further study of the R363W mutation, using restriction enzyme based analysis of 460 unrelated chromosomes in healthy Thai controls, failed to detect this mutation in the general population (data not shown), suggesting that the R363W mutation might have occurred independently in the two families, due to recurrent hydrolytic deamination of 5′ methylated cytosine resulting in transition of meCpG to TpG.

ACKNOWLEDGEMENTS

We are very grateful to Professor Keiko Kobayashi and Dr HZ Gao for preliminary mutation analysis in patient #1, which was published earlier in *Hum Mutat* 2003; 22: 24-34 (Gao *et al,* 2003). Jisnuson Svasti is a Senior Research Scholar of the Thailand Research Fund.

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