



## Case Report

## The first Mongolian cases of phenylketonuria in selective screening of inborn errors of metabolism



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## ABSTRACT

**Background:** Inborn errors of metabolism (IEM) are rare genetic disorders in which a single gene defect causes a clinically significant block in a metabolic pathway. Clinical problems arise due to either accumulation of substrates that are toxic or interfere with normal function, or deficiency of the products that are used to synthesize essential compounds. There is no report of screening results or confirmed cases of IEM in Mongolia. Only pilot study of newborn screening for congenital hypothyroidism was implemented in Mongolia, where the incidence of congenital hypothyroidism is calculated to be 1:3057 in Mongolia.

**Methods:** Two hundred twenty-three Mongolian patients, who had developmental delay, psychomotor retardation with unknown cause, seizures, hypotonia or liver dysfunction, were studied. Urinary organic acid analysis was performed in all cases using gas chromatography mass spectrometric (GC/MS) analysis. Blood amino acids and acylcarnitines were checked in the patients who had abnormal GC/MS analyses. Mutation analysis was done in the patients, who were suspected having specific inborn errors of metabolism by mass spectrometric analysis.

**Results:** One hundred thirty-nine children had normal urinary organic acid analyses. Thirty one had metabolites of valproic acid, 17 had non- or hypoketotic dicarboxylic aciduria, 14 had tyrosiluria, 12 had ketosis, 4 had elevation of lactate and pyruvate, 3 had increased excretion of urinary glycerol or methylmalonic acids, respectively, and 2 had elevation of phenylacetate and phenyllactate. We checked blood amino acids and acylcarnitines in 38 patients, which revealed phenylketonuria (PKU) in 2 patients, and one with suspected citrin deficiency. Mutation analysis in PAH was done in 2 patients with PKU, and previously reported p.R243Q, p.Y356X, p.V399V, p.A403V mutations were detected.

**Discussion:** In conclusion, these were the first genetically confirmed cases of PKU in Mongolia, and the study suggested that the newborn screening program for PKU was significant because it enabled early treatment dietary restriction, specialized formulas and other medical management for prevention of neurological handicaps in these children.

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## 1. Introduction

Inborn errors of metabolism (IEM) are rare genetic disorders caused by a single gene defect, resulting in metabolic disarrangements and neurological handicaps in children. Clinical problems arise due to accumulation of the upstream substrates which are toxic or interfere with normal function, or deficit of the products which are used to synthesize essential compounds or energy. The major groups of IEMs included organic acidemias, aminoacidopathies, fatty acid oxidation disorders,

disorders of carbohydrate metabolism, or accumulative disorders such as lysosomal diseases, and so on. First discovered were aminoacidopathies such as alkaptonuria in 1891 and phenylketonuria in 1937. When metabolic disorders are diagnosed in early infancy, treatment can commence immediately, preventing irreversible damage and life-threatening illnesses.

Initially, phenylketonuria (PKU) was screened in newborns by a bacterial inhibition assay pioneered in 1961 by Guthrie [1], and subsequently other IEMs were added to the target panel of newborn screening using tandem mass spectrometry (MS/MS). Patients with PKU show high levels of phenylalanine in the blood and consequently suffer from neurological impairment. Phenylalanine in high levels is toxic to the brain, therefore, untreated patients with PKU show mental

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retardation, convulsions, or behavioral abnormalities. In order to prevent children from such damage, the patients must be diagnosed early and follow a lifelong phenylalanine restricted diet. Munkhtuvshin et al. [19] performed the Guthrie test in 620 children aged 8 to 18 years with diagnosis of oligophrenia for purpose of detecting PKU, and 10 (1.63%) patients had elevated phenylalanine (higher than 4 mg/dl), although enzyme assay and mutation analysis were not performed (report in Mongolian).

There have never been reports confirmed cases of inborn errors of metabolism such as organic acidemias, aminoacidopathies or fatty acid oxidation disorders in Mongolia. A pilot study of newborn screening for congenital hypothyroidism was implemented in Mongolia, and the incidence of congenital hypothyroidism is calculated to be 1:3057 in Mongolia [2]. We screened urinary organic metabolites in 223 children with developmental delay and psychomotor retardation using GC/MS to determine IEM in Mongolian children.

## 2. Subjects and methods

### 2.1. Subjects

We analyzed urine samples from 223 Mongolian children (124 boys and 99 girls) who had clinical symptoms such as developmental delay, psychomotor retardation, seizures and hypotonia between 2014 and 2015. The children were from 1 day to 16 years of age. There were no patients from consanguineous marriage. 19 patients had family history of similar clinical signs and psychomotor retardation in her/his siblings or relatives. Urinary organic acid analysis was performed in all cases using GC/MS analysis, and blood amino acids and acylcarnitines levels were checked by MS/MS on the patients who had abnormality in GC/MS analysis for confirmation of fatty acid oxidation disorders. Mutation analysis was done in the patients, who were suspected of having specific inborn errors of metabolism. Informed consent for study was obtained from the parents of the patients. This study was approved by the Ethical Committee of the Ministry of Health in Mongolia.

### 2.2. Methods

#### 2.2.1. Urinary organic acid analysis.

Urinary organic acid and blood acylcarnitine analyses were performed at the Shimane University Faculty of Medicine, Shimane Prefecture, Japan. Extracts of urine in the filter paper (50 × 50 mm, Advantec 327) containing 0.2 mg of creatinine were analyzed with oximation, solvent extraction, and derivatization as described [3,4] using capillary GC/MS QP 2010 PLUS system (Shimadzu, Kyoto, Japan). The capillary column was a fused silica DB-5 column (30 m × 0.25 mm i.d.) with a 1- $\mu$ m-thick 5% phenylmethyl silicone film (J & W Scientific, Folsom, CA, USA). The internal standards comprised 20  $\mu$ g each of heptadecanoic acid (HDA) and tetracosane (C24), as well as 40  $\mu$ g of tropic acid (TA). The unit of organic acid compounds was described as relative peak area (RPA, %) to the area of internal standard (heptadecanoic acid).

#### 2.2.2. Blood amino acid and acylcarnitine analysis using MS/MS

Blood amino acids and acylcarnitines were analyzed using MS/MS (API 3000; Applied Biosystems, Foster City, CA, USA, and Shimadzu LC-MSMS 8030, Shimadzu, Kyoto, Japan). 200  $\mu$ l of methanol including an isotope-labeled internal standard (Cambridge Isotope Laboratories, Kit NSK-A/B, Cambridge, UK) was added to aliquots of blood filter paper. Supernatant aliquots were dried under a nitrogen stream, and butylated with 50  $\mu$ l of 3 N n-butanol-HCl at 65 °C for 15 min. The dried butylated sample was dissolved in 100  $\mu$ l of 80% acetonitrile:water (4:1 v/v) and then the amino acids and acylcarnitines were determined using MS/MS and quantified using ChemoView™ software (Applied Biosystems/MDS SCIEX, Toronto, Canada) [5].

### 2.2.3. Mutation analysis

Genomic DNA was extracted from the patients and their parents' peripheral blood lymphocytes using the QIAamp DNA Micro Kit (Qiagen GmbH, Hilden, Germany). Mutation analysis was performed in the Department of Pediatrics, Osaka City University Graduate School of Medicine, Japan. Thirteen sets of primers were designed for the amplification of each exon including 5' and 3' splice sites of the phenylalanine hydroxylase (PAH) gene. Exons were amplified for 35 cycles using the polymerase chain reaction (PCR) as follows: denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s with the AmpliTaq Gold PCR Master Mix (Applied Biosystems, Foster City, CA, USA) using the iCycler (Bio Rad Laboratories Inc., Hercules, CA, USA). All PCR-amplified fragments of PAH were then directly sequenced using ABI Big Dye Terminator Cycle Sequencing FS Ready Reaction Kits and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

## 3. Results

The clinical findings included 108 with developmental delay, 96 with seizures, 87 with mental retardation, 81 with hypotonia, 46 with congenital anomalies, 39 with lethargy, 37 with apnea/dyspnea, 34 with hepatomegaly, 33 with acute encephalopathy, 33 with vomiting, 15 with muscle pain, 10 with coma and 10 with cardiomyopathy, respectively.

The urinary organic acid analysis was normal in 139 children. The following metabolic abnormalities were detected in others: 31 patients who had a history of convulsions treated with valproic acid had metabolites of valproic acid, 17 had non- or hypoketotic dicarboxylic aciduria, 14 had tyrosiluria, 12 had ketosis, 4 had elevation of lactate and pyruvate, 3 had increased excretion of urinary glycerol or methylmalonic acids, respectively, and 2 had elevated levels of phenylacetic and phenyllactic acids.

We checked blood amino acids and acylcarnitines in 38 patients, who had non- or hypoketotic dicarboxylic aciduria suggesting disturbed  $\beta$ -oxidation, tyrosiluria, increased excretion of methylmalonic, phenylacetic and phenyllactic acids in urinary organic acid analysis. Blood amino acid and acylcarnitine analysis revealed PKU in 2 patients, and suspected citrin deficiency in 1 each, respectively. Acylcarnitine levels were normal in the patients who had slight elevation of methylmalonic acid in the urine. There was no patient with fatty acid oxidation disorders.

The clinical features of 2 Mongolian patients with PKU are described in the Table 1.

**Patient 1:** A girl aged 2 years and 9 months, was born as the first child of non-consanguineous parents after an uneventful pregnancy. Her initial hospitalization occurred at 9 months of age when she was hospitalized at a local hospital for seizures and treated with valproic acid. However, her epileptic episodes become more frequent and she was referred to our hospital with diagnosis of toxic encephalopathy. She had seizures, psychomotor and speech delay. Clinical examination revealed hypopigmentation (lighter skin and hair, although her parents had normal black hair, and no history of light hair on both parental sides). Complete blood count, blood glucose and electrolytes were normal. Urinary organic acid analysis using GC/MS showed marked elevation of phenylacetate (58.4%, RPA, normally no detection), phenyllactate (261.1%, RPA, normally no detection) and phenylpyruvate (379.4%, RPA, normally no detection). Blood amino acid and acylcarnitine analysis using MS/MS revealed a marked elevation of phenylalanine (1015.9 nmol/ml; cut-off level < 100 nmol/ml), low level of tyrosine (44.9 nmol/ml; normal cut-off level, <250 nmol/ml) and elevated ratio of phenylalanine/tyrosine (22.6; normal range <2.5). The patient was a compound heterozygote of a paternal p.R243Q (c.728 G to A transition) and a maternal p.V399V (c.1197 A to T transversion) in PAH gene.

**Patient 2:** A 3-year-10-month-old girl, who was born of a healthy mother. The patient had psychomotor retardation, hypotonia, behavior

**Table 1**  
Clinical and laboratory features of 2 Mongolia patients with PKU.

	Clinical diagnosis	Symptoms	GCMS result (RPA, %)	TMS result (nmol/ml)	Mutation
Patient 1	Toxic encephalopathy, epilepsy	Psychomotor retardation, epilepsy, hypopigmentation of skin and hair	Phenylacetate - 58.4 phenyllactate - 261.1 phenylpyruvate - 379.4	Phenylalanine - 1015.9 tyrosine - 44.9 Phe/Tyr - 22.6	p.R243Q p.V399V
Patient 2	Developmental delay	Psychomotor retardation, hypotonia, behavior problem, ear dysplasia, patent ductus arteriosus	Phenylacetate - 6.19 phenyllactate - 6.21 phenylpyruvate - n.d	Phenylalanine - 654.5 tyrosine - 59.6 Phe/Tyr - 10.97	p.Y356X p.A403V

problem, patent ductus arteriosus and ear dysplasia. She had normal skin and hair color. Investigation revealed normal blood counts, blood glucose and electrolytes. GC/MS analysis showed very mild detection of phenylacetate (6.21%, RPA, normally no detection) and phenyllactate (6.19%, RPA, normally no detection) without phenylpyruvate. MS/MS analysis presented mild elevation of phenylalanine (654.5 nmol/ml; cut-off level < 100 nmol/ml), low level of tyrosine (59.6 nmol/ml; cut-off level < 250 nmol/ml) and elevated ratio of phenylalanine and tyrosine (10.97; cut-off level < 2.5). The patient was a compound heterozygote of a p.Y356X (c.1068 C to A transversion) and a p.A403V (c.1208 C to T transition) in *PAH* gene. We performed genetic analysis in her mother, and detected a mutation of p.A403V. It was impossible to collect DNA sample from her father.

#### 4. Discussion

According to selective screening study for IEMs, a total detection incidence was as follows: 1:11 in Vietnam, 1:19 in India, 1:54 in China and 1:66 in Japan [6–10], and phenylketonuria, maple syrup urine disease, homocystinuria, methylmalonic and propionic acidemias were the most prevalent diseases. In this study, we found 2 patients with PKU and 1 patient with a strong suspicion of citrin deficiency in the 223 patients with clinical abnormalities. It was suggested that PKU was detected with an incidence of 1:112 by the selective screening. Similar to other countries, PKU was the most prevalent IEM although the patient number was low in this study. These were the first biochemically and genetically confirmed cases of PKU and IEM in Mongolia. Both patients with PKU already had developmental delay. This study suggests that we need to include all patients with developmental delay in selective screening for IEM. Furthermore, we should consider introducing NBS for IEM and developing specific treatment guidelines of IEMs for Mongolia. Early and presymptomatic detection and treatment intervention for patients with PKU will be essential to prevent major neurocognitive deficits [11].

The phenotypic diversity reflects heterogeneity at the molecular level, and >700 different mutations in the *PAH* gene are known to date (The Human gene mutation database). In this study, a total 4 mutations (p.R243Q, p.Y356X, p.V399V and A403V) from the 2 cases were identified in *PAH* gene. p.R243Q, p.Y356X, p.V399V mutations were previously reported in Asian patients such as China, Japan and Korea, and associated with classic PKU disease [12–15] while A403V mutation was relatively common in European population and resulted to mild hyperphenylalaninemia [16,17]. V399V looked silent mutation however it confirmed as disease causing due to splicing errors [18].

In previous report, Y356X null mutation appeared in classical and mild PKU [14] despite A403V missense mutation showed mild hyperphenylalaninemia [17] and compound heterozygotes of Y356X and A403V mutations showed very slight change in GC/MS analysis, although it was associated to mental retardation and mild PKU in our patient. The patients were the first babies in their family, and since their diagnosis were confirmed by biochemical and molecular genetic

analysis, it was possible to provide genetic counseling including a discussion of potential risks to next offspring for family planning.

In conclusion, these were the first genetically confirmed cases of PKU in Mongolia, and the study suggested that NBS program for PKU is essential to identify patients with this disease so that treatment such as restriction of dietary phenylalanine, phenylalanine free formula and other medical management for prevention of neurological handicaps can be started early.

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