Pompe disease or glycogen storage disease type II is an autosomal recessive disorder of glycogen metabolism resulting from deficiencies in the activity of the lysosomal hydrolase acid α-glucosidase in all tissues of affected individuals[1]. The clinical manifestation of Pompe disease includes a range of phenotypes, all of which involve varying degrees of myopathy. The most severe type is infantile-onset disease, with hypotonia, cardiomegaly, hepatomegaly, and death due to cardiorespiratory failure, usually before the age of 2 years[2]. The deficiency of the enzyme results in accumulation of glycogen of normal structure within lysosomes in numerous tissues, most marked in cardiac muscle, skeletal muscle, and hepatic tissues[3]. Electron microscopy reveals a specific vacuoles tightly packed with glycogen particles surrounded by a single membrane[4].

The authors report two Thai children with clinical, pathologic, and electron microscopic findings characteristic of Pompe disease. With the diagnosis, proper genetic counseling and prenatal diagnosis could be offered to the families.

MATERIAL AND METHOD

Patient 1

The patient, a boy, was born at term to a 24-year-old G1P0 Thai mother and a 30-year-old nonconsanguineous Thai father. The pregnancy and labor were uncomplicated. Birth weight was 3,100 g. He had pneumonia at age 7 months. During hospitalization, hypotonia and cardiomegaly with congestive heart failure were found. Diuretics, digitalis, and enalapril were given. He was rehospitalized 4 more times for pneumonia or congestive heart failure at ages 10, 19, 20 and 22 months. He held his head up at age 3 months, rolled over at 5 months, but was not able to sit at age 10 months. At age 22 months, he measured 75 cm (-3 SD), weighed 8.0 kg (-3 SD), and had a head circumference of 46.5 cm (-2 SD). Cardiac examination revealed a systolic murmur grade 3/6 on his left upper sternal border. His liver was palpated 5 cm below the right costal margin but the spleen was not palpable.

Electrocardiogram (EKG) showed a short PR interval, large QRS voltage, signs of left atrial dilatation and biventricular hypertrophy (Fig. 1). Roentgenograms of his chest showed marked cardiomegaly. The echocardiogram showed severe ventricular hypertrophy. Electron microscopy on a skin biopsy at age 7 months showed vacuoles filled with glycogen (Fig. 2). He died of cardiopulmonary failure with septicemia at the age of 22 months. Hemoculture was positive for *Morganella morganii*.

Patient 2

The patient, a girl, was born at full-term to a 32-year-old G1P0 Thai mother and a 37-year-old nonconsanguineous Thai father. The pregnancy was complicated by maternal gestational diabetes mellitus. The patient was born by Cesarean section with forcep extraction due to fetal distress. Birth weight was 3,600 g. APGAR scores were 6 and 8 at 1 and 5 minutes, respectively. After birth, she had dyspnea requiring hospitalization for 4 weeks. Hypotonia and cardiomegaly with congestive heart failure were found. She was hospitalized three times at ages 3, 4, and 6 months for pneumonia. At age 6 months, she could hold her head up but could not roll over. Her weight was 5.3 kg (-2.5 SD). She had respiratory distress, bilateral rhonchi on both lungs, systolic ejection murmur grade 2/6 on left sternal border, and hepatomegaly.

Her liver enzymes were elevated with alanine aminotransferase (ALT, SGPT) 89 U/L (normal: 5-45) and aspartate aminotransferase (AST, SGOT) 185 U/L (normal: 15-55). EKG showed a short PR interval, massive QRS voltage, and signs of biventricular hypertrophy. Chest roentgenograms showed striking cardiomegaly. The echocardiogram showed severe ventricular hypertrophy with low left ventricular systolic function and mild tricuspid and mitral valve regurgitation. Mitochondrial DNA analysis at position 3,243, 8,344, and 8,993 was negative. She died of cardiopulmonary failure at the age of 9 months.

At the postmortem examination, the main pathology was observed in the heart, liver, and the brain. The heart was enlarged, with the weight of 195 grams (normal: 41 ± 5). The left and right ventricular walls were thickened, and respectively measured 1.8 cm and 1.0 cm. There was also marked eccentric thickening of the interventricular septum. The liver weighed 250 g (normal: 288 ± 67), showing yellow brown cut surfaces. The brain weighed 720 g (normal: 810 ± 82). Coronal sections revealed diffusely increased firmness with gray discoloration of the white matter of both the cerebral hemispheres. The gray structures were relatively intact.

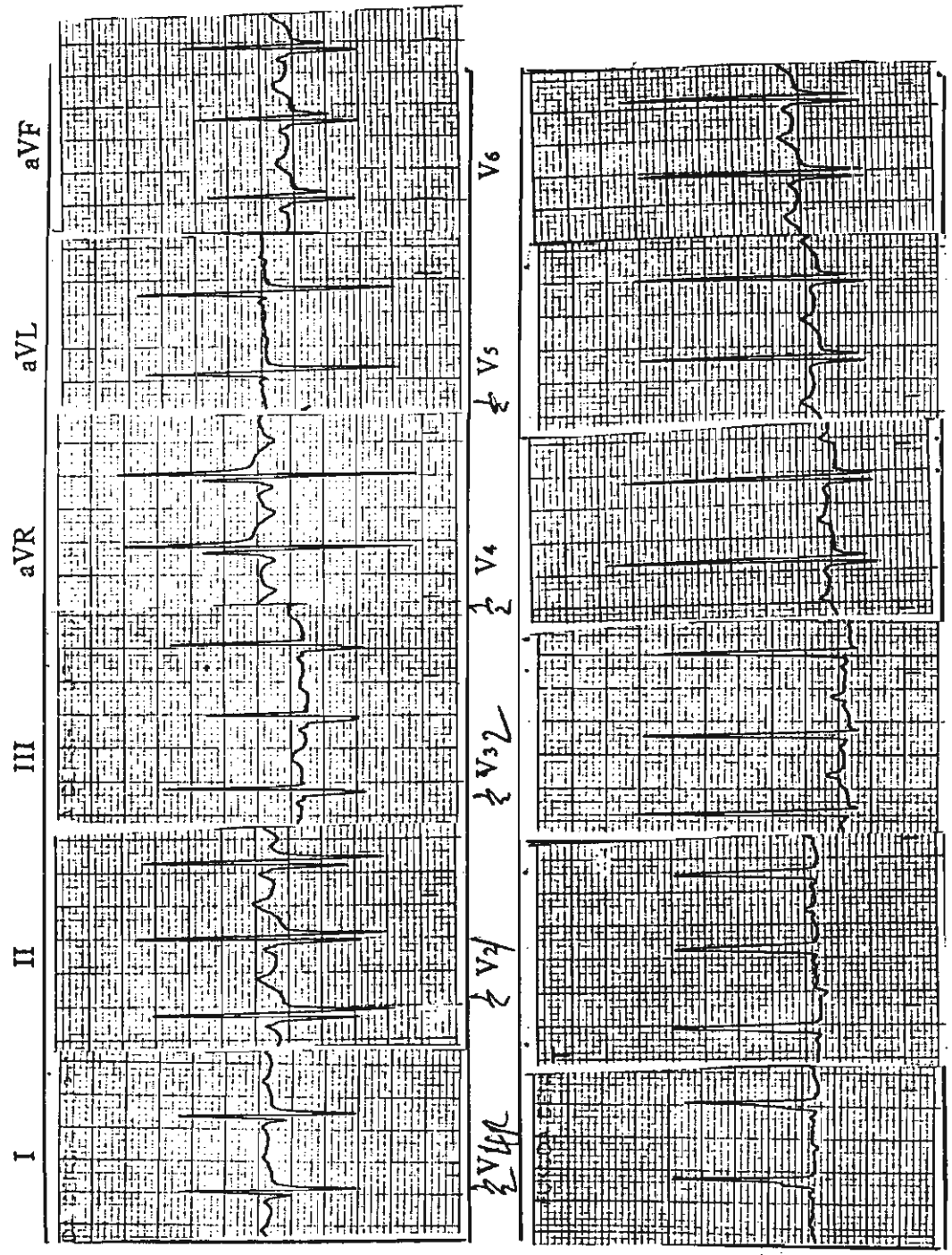

Fig. 1. The electrocardiogram of Patient 1 shows a short PR interval with large QRS complexes.

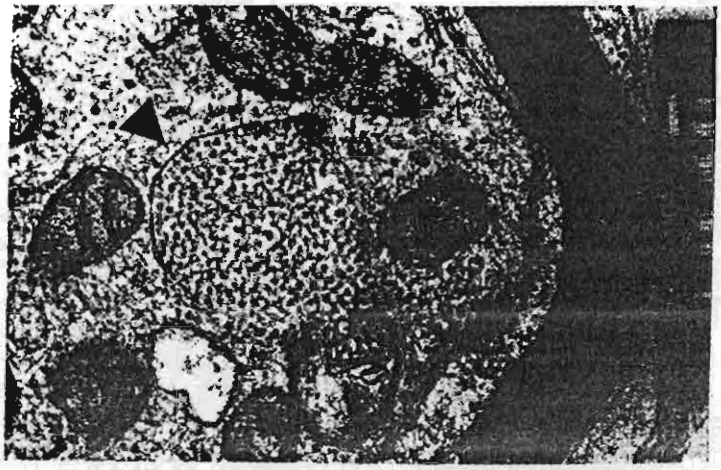

Fig. 2. Electron microscopic findings of patient 1. Note an accumulation of glycogen particles surrounded by a single membrane (arrow head).

On routine stain, virtually all cardiac muscle fibers contained cytoplasmic clear vacuoles (Fig. 3A). All of the hepatocytes were expanded with multiple small round vacuoles in the cytoplasm (Fig. 3B). Many of the neurons in the dentate nuclei of the cerebellum (Fig. 3C) and in the basal ganglia were distended with fine cytoplasmic vacuoles whereas the cortical neurons were well-preserved. Astrocytes in the white matter possessed enlarged cytoplasm, with foamy appearance (Fig. 4A). Periodic acid Schiff (PAS) staining method demonstrated PAS-positive diastase-sensitive glycogen material in the above abnormal cells, most prominent in the white matter astrocytes (Fig. 4B and 4C). Antemortem electron microscopy was performed on a skin biopsy, which demonstrated vacuoles filled with glycogen.

DISCUSSION

The authors report two Thai patients with findings characteristic of Pompe disease. One was a boy and the other a girl. Pompe disease is transmitted as an autosomal recessive trait[5]; therefore, affected individuals can be of either sex. Both of our patients had their first symptoms in their infancy; Patient 1 at age 7 months and Patient 2 in her neonatal period. Age of onset of individuals with Pompe disease varies. The most severe phenotype is the classic infantile-onset disease which presents within the first few months of life[6], even in the neonatal period[7]. The two presented patients manifested hypotonia, cardiomegaly, and congestive heart failure. Roentgenograms showed markedly enlarged hearts. EKG showed an abnormally shortened PR interval with gigantic QRS complexes. The diagnoses of Pompe disease were first suspected because of the floppy baby appearance, the cardiomegaly on chest X-rays and the findings of EKGs, which are all typical features of Pompe disease[8-10]. Patient 1 died at age 22 months and patient 2 at age 9 months due to cardiorespiratory failure, consistent with the rapidly progressive course with death usually before 2 years of age in patients with Pompe disease[2,11].

Hepatic enzymes of patient 2 were slightly elevated similar to those found in patients with Pompe disease[12]. Electron microscopic features of skin samples from both patients showed glycogen accumulations surrounded by membranes, specific for Pompe disease[4,13-15]. The intracellular vacuoles full of glycogen found in electron microscopy can be used as a rapid, safe, and reliable method for prenatal diagnosis[16]. Autopsy was performed on Patient 2. She was found to have marked accumulation of glycogen in liver, heart and numerous additional tissues including her brain. All these autopsied findings are consistent with the diagnosis of Pompe disease[3,17].

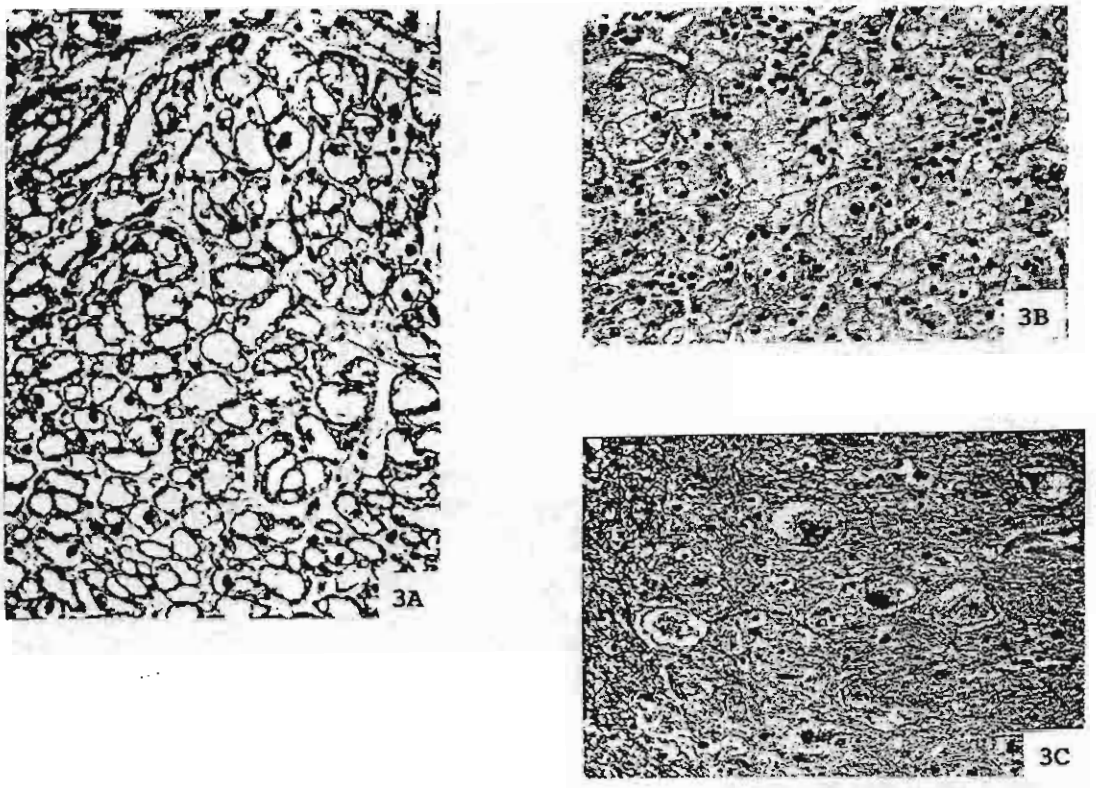

Fig. 3. Pathology of Patient 2. All of the cardiac muscle cells contain intracytoplasmic round to oval clear space (A). The liver cells are enlarged with multiple cytoplasmic vacuoles (B). Neurons in the dentate nucleus of the cerebellum are distended with cytoplasmic foamy substance, displacing the nucleus into the periphery (C), (A-C, H&E).

Even though clinical, pathological, and electron microscopic features are specific for Pompe disease, they are not pathognomonic. Cardiac abnormalities, skeletal involvement and the intravacuolar accumulation of glycogen are also found in Danon syndrome, which has normal acid α-glucosidase. Danon syndrome is inherited as an X-linked trait caused by primary deficiency of a lysosomal membrane protein, LAMP-2[18]. The diagnosis of infantile-onset Pompe disease can be confirmed by virtual absence of acid α-glucosidase in muscle biopsies or cultured fibroblasts[19-25]. Purified lymphocytes also exhibit the enzyme defects but misdiagnosis may occur with imperfectly fractionated peripheral blood lymphocytes. Assay of unfractionated leukocytes is not reliable[26-30]. Another method, which can be used to definitely diagnose patients with Pompe disease, is to perform mutation analysis. Both the cDNA and structural gene for human acid α-glucosidase have been isolated and characterized. The cDNA has 2,859 nucleotides of coding sequence predicting 952 amino acids. The structural gene contains 20 exons in approximately 20 kb of genomic DNA and has been localized to chromosome 17q25. Mutations associated with Pompe disease are various including missense mutations, nonsense mutations, deletions, and insertions[31-35]. Unfortunately, no diagnostic laboratories offering either the biochemical or molecular tests as a service for definite diagnosis of Pompe disease are available in Thailand.

A few Thai patients with Pompe disease have been reported[36-39]. The authors diagnosed two additional children during a two-year period in a single hospital suggesting that children with Pompe disease could occasionally be encountered in

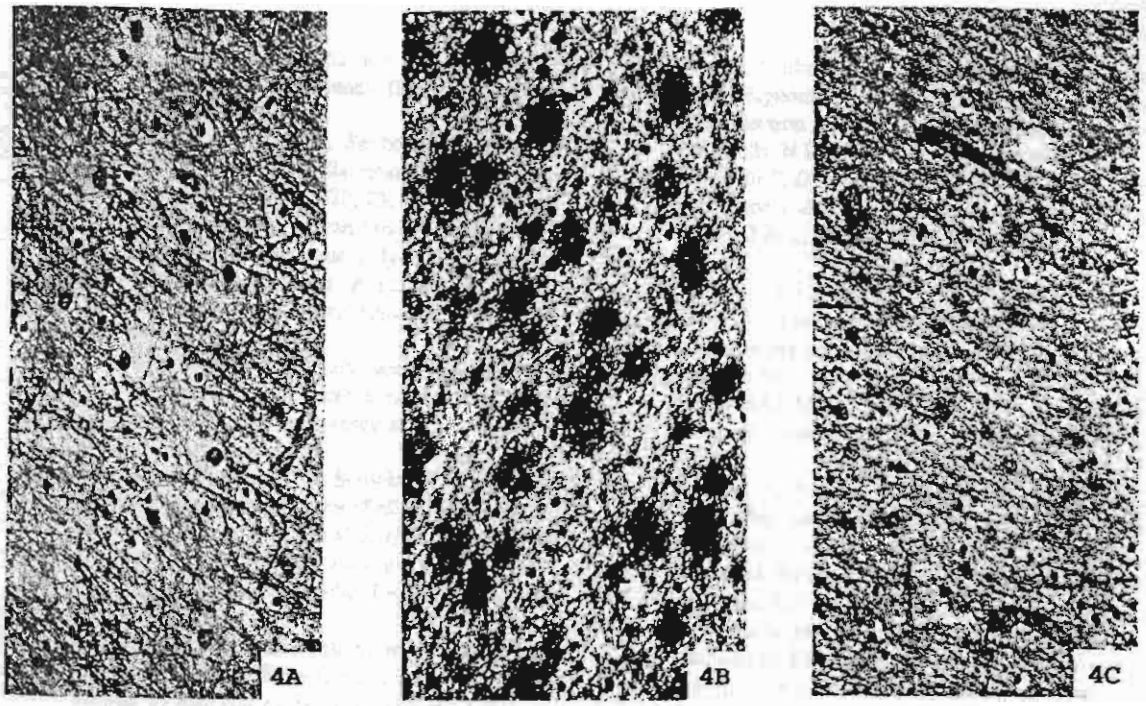

Fig. 4. Pathology of Patient 2. Astrocytes in the cerebral white matter are enlarged with foamy cytoplasm (A), which is PAS (periodic acid Schiff)-positive (B) diastase-liable (C), characteristic of glycogen (A, H&E; B, PAS; C, PAS with diastase pretreatment).

Thailand. The disease should be in the differential diagnosis for patients presenting with cardiomegaly and skeletal myopathy.

Some of the previously reported cases were definitely diagnosed by biochemical studies[36,38]. However, none of the studies were performed in Thailand. Attempts to establish the biochemical or molecular studies to definitely diagnose Pompe disease in Thailand should be encouraged. With the definite diagnosis, proper genetic counseling and prenatal diagnosis could be offered to the families.

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ลักษณะทางคลินิก พยาธิสภาพและกล้องจุลทรรศน์อิเลคตรอนของเด็กไทยซึ่งป่วยด้วยโรคปอมเปสองราย

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รายงานผู้ป่วยเด็กชายไทย 1 ราย เริ่มมีกล้ามเนื้ออ่อนแรง หัวใจโต และตับโต ตั้งแต่อายุ 7 เดือน และเด็กหญิงไทย 1 ราย มีอาการเช่นเดียวกันตั้งแต่ช่วงทารกแรกเกิด การตรวจทางรังสีของผู้ป่วยทั้งสองรายพบหัวใจโต, คลื่นไฟฟ้าหัวใจมีช่วง PR สั้น, QRS complexes ใหญ่ และการตรวจทางกล้องจุลทรรศน์อิเลคตรอนของชิ้นผิวหนังพบไกลโคเจนสะสมอยู่ในอวัยวะเซลล์ ผู้ป่วยเสียชีวิตด้วยระบบหัวใจและหายใจล้มเหลวขณะอายุ 22 เดือนและ 9 เดือนตามลำดับ การชันสูตรศพของผู้ป่วยหญิงพบมีไกลโคเจนสะสมอยู่ในตับ กล้ามเนื้อหัวใจ และเนื้อเยื่ออื่น ๆ รวมทั้งสมองลักษณะทางคลินิก, พยาธิสภาพ, และกล้องจุลทรรศน์อิเลคตรอนของผู้ป่วยทั้ง 2 รายเข้าได้กับโรค Pompe

โรค Pompe เป็นโรคที่มีการถ่ายทอดทางพันธุกรรมแบบยีนด้อย เกิดจากความผิดปกติของ acid α-glucosidase ในไลโซโซม ซึ่งเป็นส่วนหนึ่งของกระบวนการเมแทบอลิซึมของไกลโคเจน การวินิจฉัยที่แน่ชัดทำได้โดยการตรวจระดับการทำงานของเอ็นไซม์หรือการตรวจหาการกลายพันธุ์ อย่างไรก็ตามยังไม่มีห้องปฏิบัติการในประเทศไทยที่ให้บริการการตรวจนี้ เนื่องจากมีรายงานผู้ป่วยโรค Pompe ในประเทศไทยอยู่ประปราย จึงควรมีการสนับสนุนให้มีการตรวจทางห้องปฏิบัติการดังกล่าวเพื่อให้สามารถให้การวินิจฉัยที่แน่ชัดแก่ผู้ป่วยได้ ทั้งนี้เพื่อประโยชน์ในการให้คำปรึกษาแนะนำทางพันธุศาสตร์และการวินิจฉัยก่อนคลอด

คำสำคัญ : โรคสะสมไกลโคเจน, โรคปอมเป่, จุลทรรศน์อิเลคตรอน

วรศักดิ์ โชติเลอศักดิ์, ชนพ ช่วงโชติ, ไพโรจน์ โชติวิทยธารากร, และคณะ
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บทความที่ 6

ESTABLISHING GAS CHROMATOGRAPHY - MASS SPECTROMETRY TO DIAGNOSE ORGANIC ACIDEMIAS IN THAILAND

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Abstract. Disorders of organic acid metabolism are a group of disorders which has long been ignored by majority of Thai physicians. Part of this is due to lack of laboratories in Thailand to verify the diagnosis of the disorders. We have recently developed a technique to qualitatively analyze organic acids utilizing Gas Chromatography - Mass Spectrometry (GC-MS). Eight patients in four families were successfully identified as having organic acidemias (OA) by this method. Two families had methylmalonic acidemia, one had propionic acidemia, and the other had 3-methylcrotonyl CoA carboxylase deficiency. To our knowledge, this is the first laboratory in Thailand being able to use GC-MS to diagnose OA. Availability of a laboratory in Thailand and affordability of the test are expected to result in earlier diagnosis and identification of more cases of OA in Southeast Asian countries. Consequently, prompt and proper treatment can be anticipated which should lead to better prognosis for patients with this group of disorder.

INTRODUCTION

Patients presented with lethargy, hypotonia, hypertonia, tachypnea, seizures, ataxia, vomiting, failure to thrive, delayed development, and hepatomegaly may have organic acid disorders. Abnormal clinical chemistries such as cytopenia, metabolic acidosis, hyperammonemia, hypoglycemia, lactic acidemia, ketosis may also suggest abnormalities of organic acid metabolism (Goodman, 1996; Clarke, 1996). However, this group of disorders has long been ignored by many of the Thai physicians. Part of which may be due to unavailability of laboratories in Thailand to verify the diagnosis of the disorders.

The qualitative analysis of organic acids by gas chromatography - mass spectrometry (GC-MS) has well established as an important method for the diagnosis of disorders of organic acid metabolism since early 1980s (Sweetman, 1991). Here we reported accomplishment of utilizing GC-MS to identify organic acids and making diagnoses of patients with methylmalonic acidemia, propionic acidemia, and 3-methylcrotonyl CoA carboxylase deficiency. This will expedite the diagnosis of OA in Thai and other Southeast Asian patients. Therefore, prompt treatment and better prognosis can be anticipated.

MATERIALS AND METHODS

Urine organic acid analysis using GC-MS

Three drops of 6N HCl were added to 1 ml of urine or of 80 mg/100 ml control substrates (Table 1). NaCl was added until saturated. Then, 1 ml of ethylacetate, as a solvent to extract OA, was added. After the solution was mixed and centrifuged at 3,000 rpm for 3 minutes, the upper layer was transferred to a new tube and evaporated with nitrogen gas from N-evaporator till dry. We repeated extraction of organic acids two more times, each with 1 ml of ethylacetate. When it dried, BSTFA-TMCS [(N,O-bis (trimethylsilyl) trifluoroacetamide-trimethylchlorosilane) (Supelco, PA, USA)] 100 µl was added, then mixed, and heated at 90°C

Table 1
Standards used and their retention times.

No	Standards	Retention times (min)
1.	Methylmalonic acid	9.50
2.	Adipic acid	19.93
3.	Succinyl acetone	23.31, 24.55, 25.44
4.	Orotic acid	28.57
5.	Sebacic acid	33.22
6.	Undecanedioic acid	36.61

in a water bath for 10 minutes. The sample was then injected into the GC (HP 5890 series II PLUS) using Helium as carrier gas with the flow rate of 0.5 ml/minutes. The column used was HP-Ultra2, 25 m x 0.2 mm x 0.33 μm. The injection condition was "split (20:1), inlet at 250°C". The oven temperatures were 100°C for 1 minute, then increased with the rate of 3°C per minute to 250°C and sustained for 1 more minute. The substances were detected by mass selective detector (Hewett Packard 5972 series) at 280°C and were identified by a library kindly provided by Dr George Thomas of the Kennedy Kreiger, USA and Dr Tina Cowan at the University of Maryland, USA.

Patients

Family 1: Patient 1 was born at 37 weeks of gestation. The pregnancy, labor and delivery was unremarkable. His parents were second cousins (see pedigree in Fig 1). The patient's older brother (patient 2) died of hypoglycemia and severe metabolic acidosis at age 5 months. Patient 1 suffered from persistent pulmonary hypertension and pneumonia requiring ventilatory support for the first 16 days of life. Because his older brother was suspected of having an organic acidemia, the patient was given carnitine 300 mg/kg BW/day since the first week of life. At age 3 weeks, his general condition improved and he was discharged from the neonatal intensive care unit. The carnitine was discontinued and he was fed on regular formula. At age 2 months, he developed lethargy. Physical examination revealed mild dehydration, jaundice, and tachypnea. Laboratory data demonstrated pancytopenia with hemoglobin 9.52 g/dl, hematocrit 28.3%, white blood cell count 1,090 cells/mm^3, and platelet 19,300 /mm^3. Urine pH was 6 and urine ketone 2+. He did not have hypoglycemia. Serum sodium was 133 mEq/l, potassium 4.4 mEq/l, chloride 94 mEq/l, bicarbonate 17 mEq/l and the anion gap of 22 mEq/l. BUN was 6 mg/dl, Cr 0.5 mg/dl, total bilirubin 6.46 mg/dl, direct bilirubin 5.71 mg/dl, alkaline phosphatase 220, SGOT 69 U/l, SGPT 95 U/l, ionized calcium 1.33 mmol/l (normal range 1.15-1.8 mmol/l), magnesium 2.0 mg/dl (normal range 1.6-2.6 mg/dl), and ammonia 492 μg/dl (normal range: 25-94 μg/dl). Urine ferric chloride test and DNPH tests were negative. Urine p-nitroaniline test was positive.

Family 2: An 8-month-old boy (patient 3) presented with fever, vomiting and lethargy. He was born to a G3P2 27-year-old mother and a 32-year-old unrelated father. The pregnancy and delivery were unremarkable. He had an older brother (patient 4) who died at age 2 years because of severe acidosis. Another older sister had been normal. On physical examination, he was tachypneic, lethargic, and moderately dehydrated. Blood cell counts were within normal limits. Serum sodium was 134 mEq/l, potassium 2.5 mEq/l, chloride 98 mEq/l, bicarbonate 11 mEq/l and the anion gap 25 mEq/l. BUN was 2 mg/dl and Cr 0.6 mg/dl. Liver function tests were unremarkable. Ammonia level was 350 μg/dl (normal range: 25-94 μg/dl). Urine ferric chloride test and DNPH tests were negative. Urine p-nitroaniline test was positive.

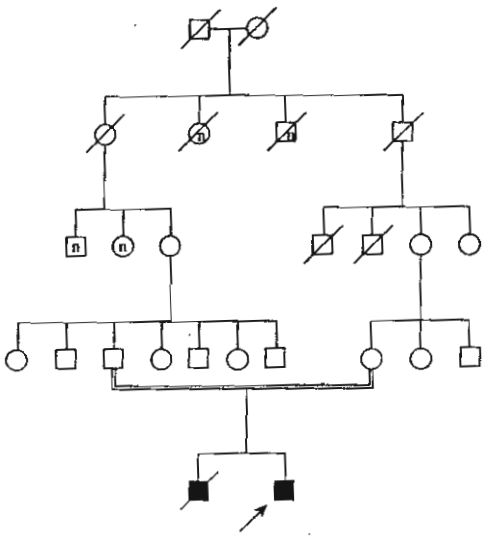

Fig 1–Pedigree of family 1.

Family 3: A one-month-old boy (patient 5) presented with lethargy for 3 days before admission. His parents were second cousins. His older brother (patient 6) died at age 3 months from severe metabolic acidosis without a definite diagnosis. However, the mother recognized the similar manifestations in her 2 children. Upon admission, his body weight was 2,700 g (his birth weight was 3,200 g). Physical examination revealed moderate dehydration and hepatomegaly with palpable liver 2 cm below his right costal margin. Laboratory data showed severe metabolic acidosis with initial bicarbonate of 6 mEq/l. The sodium was 132 mEq/l, potassium 4.8 mEq/l, and chloride 100 mEq/l. The blood sugar was 78 mg/dl, BUN 18 mg/dl, and Cr 0.8 mg/dl. The ammonia level was 600 μg/dl. Urine examination showed pH of 5.5, specific gravity 1.026, ketone 2+ and negative for protein and sugar.

Family 4: A 3-year-old girl (patient 7) presented with upper respiratory tract infection. She was born at term following an uncomplicated pregnancy, labor, and delivery. She was admitted once at age 8 months due to respiratory tract infection. Her parents were not consanguineous. Her brother (patient 8) had died of severe metabolic acidosis at age 1 year and 2 months. Physical examination of patient 7 revealed moderate dehydration and tachypnea. Her blood counts were within normal limits. Urine pH was 5 and urine ketone was 4+. She had severe metabolic acidosis with serum sodium of 139 mEq/l, potassium 4.6 mEq/l, chloride 109 mEq/l, bicarbonate 2 mEq/l and the anion gap 28 mEq/l. Her blood sugar was 77 mg/dl, BUN 8.21 mg/dl, Cr 0.54 mg/dl, ammonia 86 μM (normal range: 9-33 μM), and lactate 5.8 mM (normal range: 0.89-2.09 mM). Urine ferric chloride test and urine reducing substance test were negative. Plasma and urine amino acid analyses were unremarkable.

RESULTS

Urine organic acid analysis

All substances used as controls were retrieved and correctly identified by the libraries. Table 1 illustrated retention times for each substance. Fig 2 (A-E) demonstrate tracing of the substances.

Patients

Urine organic acid analysis of the patient 1 from family 1 (Fig 3A) revealed large amounts of 3-hydroxypropionate and methylcitrate. Small peak of 3-OH isovalerate was present. The pattern was consistent with propionic acidemia. Urine samples of patient 3 from family 2 (Fig 3B) and patient 5 from family 3 (Fig 3C) revealed huge peaks of methylmalonic acid, which was diagnostic for methylmalonic acidemia. Urine organic analysis of the patient 7 from family 4 (Fig 3D) revealed large amounts of 3-methylcrotonylglycine and 3-hydroxyisovalerate. The pattern was consistent with 3-methylcrotonyl CoA carboxylase deficiency.

DISCUSSION

In 1966, isovaleric acidemia, the first organic acidopathy was described. Since then, more than 50 phenotypically different organic acidemias are

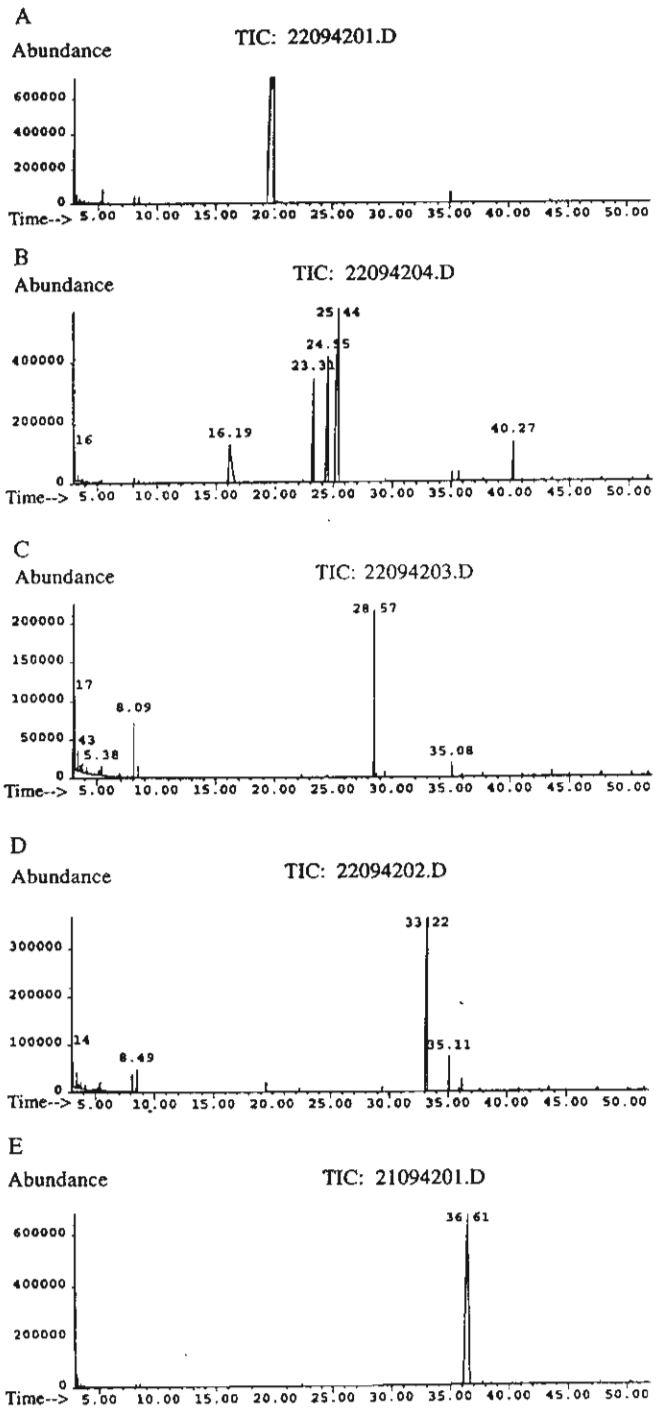

Fig 2–tracings of adipic acid (A), succinyl acetone (B), orotic acid (C), sebacic acid (D), and undecanedioic acid (E) as internal standard.

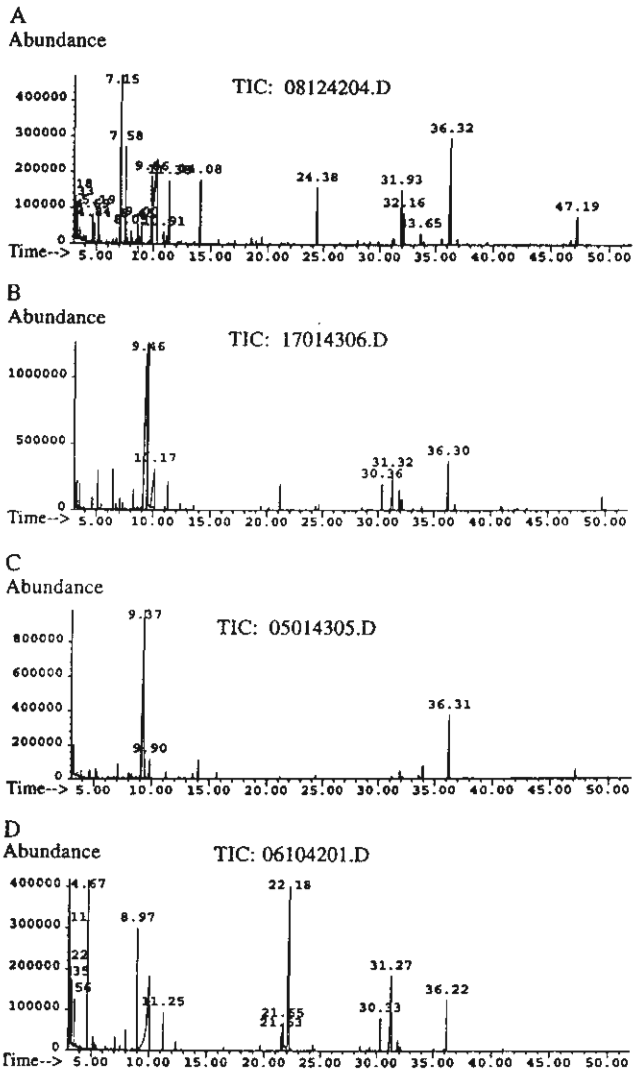

Fig 3–Urine organic acid tracing of patient 1 (A), patient 3 (B), patient 5 (C), and patient 7 (D). Panel A showed large peaks of 3-hydroxypropionate at 7.15 minutes and methylcitrate at 31.93 and 32.16 minutes. A small peak of 3-OH isovalerate was present at 9.00 minutes. The peak at 36.32 minutes was the undecanedioic acid added as an internal standard. The pattern was consistent with propionic acidemia. Panel B and C revealed huge peaks of methylmalonic acid at 9.46 and 9.37 minutes, respectively. The peaks at 36.30 minutes in panel B and at 36.31 minutes in panel C were the internal standard. They are diagnostic for methylmalonic acidemia. Panel D revealed large peaks of 3-methylcrotonylglycine at 21.65 and 22.18 minutes and 3-hydroxyisovalerate at 8.97 minutes. The peak at 36.22 minutes was the internal standard. The pattern was consistent with 3-methylcrotonyl CoA carboxylase deficiency.

Table 2
Indications for urine OA analysis.

1. Acute, chronic or recurrent metabolic acidosis, with or without an anion gap, hypoglycemia or hyperammonemia especially when induced by protein intake or infection.
2. Episodic neutropenia, and thrombocytopenia when associated with ketoacidosis.
3. Unusual odor.
4. Childhood onset of progressive extrapyramidal disease.
5. Reye syndrome when recurrent, familial, or in infancy.
6. Neurologic syndrome with alopecia and rash.

identified (Ozand, 1991). Several symptoms suggest OA and some metabolic screening tests are helpful (Buist, 1995). Table 2 illustrates some indications for urine OA analysis with GC-MS.

In developed countries, GC-MS is the most common method used to diagnose the disorders. Although GC-MS has long been available in Thailand, it has been mainly used to identify medications such as anticonvulsants, and illicit drugs, for examples, heroin. Unfortunately, it had never been utilized to diagnose OA. In collaboration between Department of Pediatrics and Department of Forensic Medicine of King Chulalongkorn Memorial Hospital, we modified a method currently used at the Kennedy Krieger Institute at Baltimore, MD, USA for diagnosing OA in Thailand. The important steps are isolation of the organic acids from physiological fluids, formation of volatile derivatives, and GC-MS analysis. Isolation of the acids is commonly accomplished by solvent extraction, which is ethylacetate in this case. Volatile trimethylsilyl (TMS) derivatives are the most useful and versatile for the wide range of chemical groups in organic acids. They formed by heating with bis-trifluoracetamide (BSTFA). The capillary columns have an excellent capacity to handle the wide range of acid concentrations. Then, unambiguous identification of compound is made by mass spectra.

Collection of urine specimens from patients has to be careful. The urine should be collected during the metabolic derangement. Urine during normal periods usually provides no abnormal acids and gives a false negative result. Urine should be collected in containers without preservatives and

frozen as soon as possible. Then the samples can be stored at -20°C until analyzed. Making a diagnosis of an organic acidemia is by identification of abnormal organic acids not present in urine of normal individuals. Therefore, even this method is a qualitative assay, it is very powerful and has few problems in making diagnosis.

Previously, there were few reported cases of organic acidemias in Thai patients (Wasant, 1995). In addition, they were diagnosed either by metabolic screening tests performed in Thailand or by GC-MS performed in developed countries. Here, using our newly developed technique, we were able to identify 4 more families with organic acidemias. Two had methylmalonic acidemia, one had propionic acidemia, and the other had 3-methylcrotonyl CoA carboxylase deficiency.

Each of our 4 families had 2 affected siblings. Even though none of the urine samples of the first child in each family were available and analyzed, we believe they had the same disorders as their younger siblings because of their similar clinical and laboratory data. Two of our four families had history of consanguineous marriage emphasizing the autosomal recessive pattern of inheritance in these metabolic disorders.

Availability of a laboratory in Thailand and affordability of the test are expected to result in earlier diagnosis and identification of more cases of OA. Therefore prompt and proper treatment can be anticipated which should lead to better prognosis for patients with this group of disorder.

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บทความที่ 7

A Thai Boy with Hereditary Enzymopenic Methemoglobinemia Type II

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Abstract

Individuals with methemoglobin exceeding 1.5 g/dl have clinically obvious central cyanosis. Hereditary methemoglobinemia is due either to autosomal dominant M hemoglobins or to autosomal recessive enzymopenic methemoglobinemia. Four types of enzymopenic methemoglobinemia have been described. In addition to methemoglobinemia, individuals with type II, which is the generalized cytochrome b_5 reductase deficiency, have severe and progressive neurological disabilities.

Here we report a 3-year-old Thai boy with type II hereditary enzymopenic methemoglobinemia. He was born to a second-cousin couple. His central cyanosis was first observed around 10 months of age. His neurological abnormalities were seizures beginning at 1 year of age, microcephaly, and inability to hold his head up. His cardiovascular and pulmonary evaluations were unremarkable. Methemoglobin level by spectral absorption pattern was 18 per cent. A qualitative enzymatic assay confirmed the deficiency of the cytochrome b_5 reductase enzyme. With this definite diagnosis, a prenatal diagnosis for the next child of this couple will be possible.

Key word : Methemoglobinemia, Cytochrome b_5 Reductase

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Central cyanosis is most commonly due to cardiopulmonary diseases[1]. If evaluations of the cardiovascular and pulmonary systems are unremarkable, other disorders should be considered such as those of the central nervous system causing hypoventilation and hematological disorders. One of the hematological causes is methemoglobinemia[2].

Oxygen transport depends on the maintenance of hemoglobin in the ferrous (reduced, Fe^{2+}) state. Methemoglobin is hemoglobin in which the iron has been oxidized from the ferrous to the ferric (oxidized, Fe^{3+}) state and is incapable of binding oxygen[3]. Normal erythrocytes contain less than 1 per cent methemoglobin. As red cells circulate, a small amount of hemoglobin autooxidizes to methemoglobin. The methemoglobin formed is normally reduced by cytochrome b_5 and cytochrome b_5 reductase[4] (Fig. 1). If methemoglobin exceeds 1.5 g/dl, affected individuals will have clinically obvious central cyanosis[5]. Etiologically, methemoglobin can either be acquired or is hereditary. Acquired methemoglobinemia is generally due to exposure to certain drugs or toxins such as nitrites, nitrates, and sulfonamides[6]. Hereditary methemoglobinemia is due either to the presence of one of the M hemoglobins or to the deficiency of cytochrome b_5 or the enzyme cytochrome b_5 reductase[7].

Here we report a 3-year-old Thai boy with central cyanosis and delayed development born to a couple who were second cousins. His cyanosis was shown to be caused by methemoglobinemia as determined by a spectral absorption pattern. The etiology of the methemoglobinemia was cytochrome b_5 reductase deficiency, as confirmed by a qualitative enzymatic assay.

MATERIAL AND METHOD

Patient

A Thai boy was born to a 21 year-old G_2P_1 mother and a 24 year-old father. The parents were second cousins (see pedigree in Fig. 2). There was no known exposure to teratogenic agents, infections, or other environmental hazards. Pregnancy, labor and delivery were normal. His birth weight was 2,800 g. With no complications, he was discharged from hospital 3 days after birth.

The child presented to another hospital at the age of 6 months because of delayed development. At that time, he could smile but was not able to hold his head up. Physical examination showed microcephaly. Radiographs of his skull revealed a small cranial vault with normal sutures and no abnormal calcification. He was subsequently admitted to hospital at the age of 7 months with a diagnosis of measles, pneumonia and diarrhea. He was not cyanotic at that time. He was then lost to follow-up.

At the age of 10 months, the patient was taken to Chulalongkorn Hospital for the first time due to rhinorrhea. He was still unable to control his head. His weight was 5,540 g (-4SD) and his anterior fontanel was closed. Central cyanosis was observed for the first time. Examination of the heart, lungs, and abdomen were within normal limits. No finger clubbing was noted. Oxygen satura-

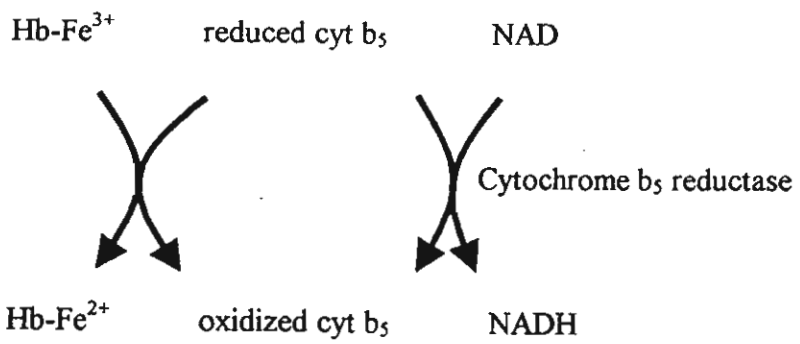

Fig. 1. Erythrocyte pathways for reduction of methemoglobin.

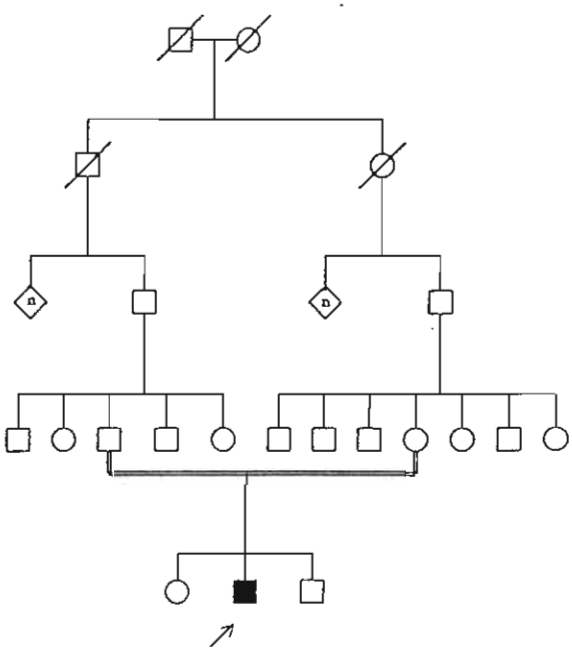

Fig. 2. Pedigree.

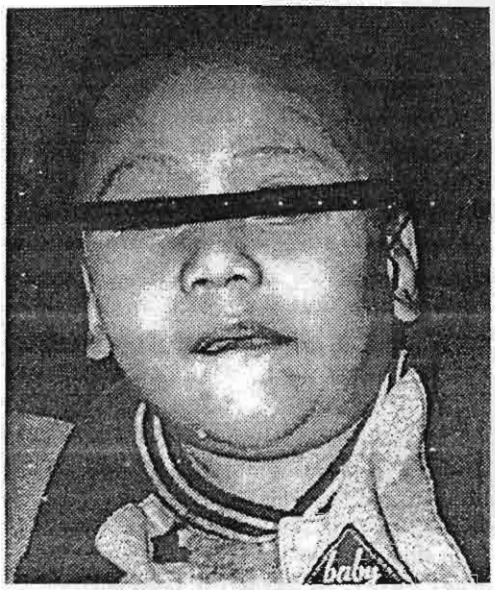

Fig. 3. The patient's face.

tion by pulse oxymetry at room temperature was 96 per cent and with 10 liters per minute of oxygen was 97-98 per cent. Laboratory data showed a hemoglobin concentration of 12.3 g/dl, hematocrit 37.3 per cent, white blood cells 9,090 /mm^3, neutrophils 50 per cent, lymphocytes 39 per cent, monocytes 5 per cent, atypical lymphocytes 4 per cent, eosinophils 2 per cent, and platelet 259,000 / mm^3. The mean corpuscular volume (MCV) was 68.5 fL, mean corpuscular hemoglobin (MCH) 22.7 mmol/L, and mean corpuscular hemoglobin concentration (MCHC) 33.1 fmol/cell. Plasma glucose was 86 g/dl, BUN 6 mg/dl, Cr 0.4 mg/dl, sodium 137 mEq/L, potassium 4.9 mEq/L, chloride 105 mEq/L, and bicarbonate 19 mEq/L. The parents declined any further investigations and did not bring the patient for follow-up.

At 3 years of age, the patient was admitted for investigation of central cyanosis, delayed development and seizures. He was still unable to hold his head up. His first seizure occurred at around 1 year of age and the frequency of the seizures had increased to a few times a day during the 3 months before admission.

Physical examination revealed an alert Thai boy with circumoral and peripheral cyanosis without respiratory distress (Fig. 3). His body weight was 7.5 kg (-2 SD), length 75 cm (-2 SD), head circumference 42.5 cm (-5 SD), body temperature 36.8°C, respiratory rate 22/min, and pulse rate 105/min. Blood pressure of his right arm, left arm, right thigh and left thigh were 87/39, 96/34, 107/41 and 109/47 mmHg, respectively. He was not pale or icteric. Examination of his chest showed normal contour, no retraction, and normal breath sounds. His heart sounds were normal with no cardiac murmur. The liver and spleen were not enlarged. His genitalia were normal for a prepubertal male. No finger clubbing was observed. Neurological examination revealed normal cranial nerves, normal power but increased tone of muscles of all extremities, normal response to pain stimuli, reflex 3+, plantar response to Babinski test, no clonus, and no signs of meningeal irritation.

Laboratory data showed a hemoglobin concentration of 10.2 g/dl, hematocrit 32.9 per cent, white blood cells 8,930 /mm^3, neutrophils 67 per cent, lymphocytes 23 per cent, monocytes 8 per cent, atypical lymphocytes 1 per cent, eosinophils 1 per cent, and platelet 356,000 /mm^3. The MCV was 63.9 fL, MCH 19.8 mmol/L, and MCHC 31.0 fmol/cell. Peripheral blood smear revealed anisocytosis 1+ and hypochromic microcytic red cells 2+.

Urine analysis showed specific gravity of 1.037, protein 1+, glucose –ve, and no cells. Plasma glucose was 104 g/dl, BUN 17 mg/dl, Cr 0.6 mg/dl, calcium 9.9 g/dl, sodium 144 mEq/L, potassium 4.3 mEq/L, chloride 112 mEq/L, and bicarbonate 18 mEq/L. A chest radiograph revealed a normal cardiac shadow and pulmonary blood flow. An echocardiogram revealed no intracardiac or intrapulmonary shunts. All cardiac valves appeared normal. Oxygen saturations by pulse oxymetry at room air and at the time of receiving 10 L/min of oxygen were around 90 per cent. Arterial blood gas taken at the time of receiving 10 L/min of oxygen revealed pH of 7.42, pO_2 149.9 mmHg, pCO_2 27.2 mmHg, HCO_3^- 17.7 mEq/L and SpO_2 99 per cent. The direct measurement of oxygen saturation at that time was 84 per cent. Glucose-6-phosphate dehydrogenase activity was normal.

Screening for methemoglobin

Three ml of peripheral blood was drawn from the patient and a control subject. The color of the blood from the patient was chocolate brown while that from the control was dark red. When mixed with oxygen, the patient's blood specimen remained a chocolate brown but that of the control changed to a red color.

Methemoglobin level

Direct measurement of methemoglobin by a spectral absorption pattern[8] using a spectrophotometer revealed a methemoglobin concentration of 18 per cent.

Qualitative enzymatic assay

Five ml of peripheral blood was drawn from the patient and a control subject. A qualitative enzymatic assay of cytochrome b_5 reductase (methemoglobin reductase) was performed by measuring the rate of defluorescence of reduced NAD (NADH) in a reduction reaction of dichlorophenol-indophenol (DCIP) as previously described [9]. The principle of the test is illustrated in figure 4. The control specimen was defluorescent around 30 minutes while the patient's specimens were not defluorescent until 80 minutes. The result was interpreted by a scientist who had been blinded to the specimen identity. Prolongation of the defluorescence suggested deficiency of the cytochrome b_5 reductase system.

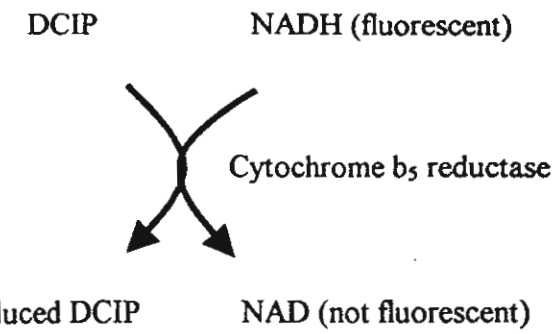

Fig. 4. Principle of the qualitative assay of the activity of the cytochrome b_5 reductase. After adding whole blood to a hemolyzing agent, NADH, and DCIP, in the presence of cytochrome b_5 reductase in the red cells, the DCIP is reduced by NADH. During the reaction, NADH, which fluoresces when illuminated by long wavelength UV light, is oxidized to NAD, which is not fluorescent.

Therapeutic trial

The patient was given 1 per cent methylene blue 0.8 ml intravenously twice daily (2 mg/kg/day). The cyanosis disappeared within 24 hours after starting the methylene blue. Arterial blood gas at that time showed a pH of 7.327, pO_2 107.4, pCO_2 30.2, HCO_3^- 15.3, and SpO_2 97.4 per cent. Direct measurement of oxygen saturation showed SpO_2 of 95.9 per cent. After the discontinuation of the methylene blue, the cyanosis reappeared.

DISCUSSION

This patient came to medical attention because of cyanosis and developmental delay. Because there was no evidence of heart or lung disease, methemoglobinemia was considered. One of the simple bedside procedures to determine methemoglobinemia was performed. After mixing a blood specimen with air or oxygen, if the cyanosis is due to decreased oxygen saturation, it will change from a purple to a red color. In contrast, a blood specimen from this patient remained a chocolate brown color despite exposure to oxygen. This finding suggested methemoglobinemia, which was later confirmed by spectroscopic examination of the hemolysate. The patient's

methemoglobin level was 18 per cent, which was several times higher than that of a normal individual. Moreover, the diagnosis of methemoglobinemia was strengthened by the disappearance of the cyanosis and the increase of SpO_2 after administration of methylene blue (from 84% to 95.9%) and by the reappearance of the cyanosis after discontinuation of the medication.

Oxygen saturation can be determined by several methods. Pulse oxymetry measures the transmission of 2 wavelengths of light most absorbed by oxyhemoglobin and deoxyhemoglobin. A blood gas machine calculates oxygen saturation from the partial pressure of oxygen in the blood[10]. Therefore, the values of oxygen saturation obtained by pulse oxymetry and a blood gas machine are unreliable in the presence of methemoglobin. The oxygen saturation in this patient measured by pulse oxymetry and a blood gas machine was not less than 90 per cent, whereas, direct measurement revealed an oxygen saturation of only 84 per cent. Clinicians should be aware of the unreliability of oxygen saturation determined by pulse oxymetry and a blood gas machine when an abnormal hemoglobin is present.

A small proportion of hemoglobin autooxidizes when red cells circulate. The methemoglobin formed is normally reduced by the reactions shown in Fig. 1. The major pathway of methemoglobin reduction is catalyzed by cytochrome b_5 and cytochrome b_5 reductase[11]. With the capacity to reduce methemoglobin far exceeding the normal rate of hemoglobin oxidation, the steady-state level of methemoglobin in normal red cells is less than 1 per cent[12]. Etiologies of methemoglobinemia can be classified into 2 major classes: acquired and hereditary. Acquired methemoglobinemia is generally due to exposure to certain drugs or toxins, which can be life-threatening. Nitrite and chlorate oxidize the heme iron directly. Aniline dyes, acetanilide, sulfonamides and lidocaine are other examples of compounds causing clinically significant methemoglobinemia[13].

Hereditary methemoglobinemia is due either to the presence of one of the M hemoglobins or to deficiency of the enzyme cytochrome b_5 reductase or cytochrome b_5. The M hemoglobins are hemoglobin variants having amino acid substitutions of residues responsible for the binding of the heme iron to the globin, which facilitates the oxidation of the heme iron in the affected subunit[14]. There have been 5 variants described, two of which, Hb M Boston and Hb M Iwate, are a-chain variants. In these cases, patients are cyanotic at birth. Individuals with the other three ß-chain variants, Hb M Saskatoon, Hb M Hyde Park, and Hb M Milwaukee, do not become cyanotic until about 4 to 6 months of age, when fetal hemoglobin has been replaced by adult hemoglobin. Except for cyanosis, these patients are asymptomatic. All of the variants are inherited in an autosomal dominant manner.

The other variety of hereditary methemoglobinemia is the enzymopenic form, which is caused by the deficiency of either the cytochrome b_5 reductase or the cytochrome b_5. This condition is inherited in an autosomal recessive pattern[15]. Patients have lifelong cyanosis of variable degree, depending on the level of methemoglobin. Untreated individuals usually have 15 to 30 per cent methemoglobin. The patient described in this report had 18 per cent methemoglobin and the qualitative enzyme assay for cytochrome b_5 reductase revealed decreased activity. In addition, the history of his parent's consanguineous marriage is consistent with the recessive mode of inheritance.

The enzymopenic hereditary methemoglobinemia has been classified into 4 types based on clinical and biochemical features[15]. The most common is type I, in which the deficiency of cytochrome b_5 reductase is limited to the erythrocytes. These subjects have methemoglobinemia alone without other symptoms[10]. Type II is the generalized form and occurs in 10 to 15 per cent of cases. Cytochrome b_5 reductase is deficient in all tissues. In addition to methemoglobinemia, patients with this type have severe and progressive neurological disabilities[16-18]. In type III, cytochrome b_5 reductase deficiency is limited to hematopoietic cells and is demonstrable in red cells, lymphocytes, granulocytes, and platelets. The only clinical manifestation is cyanosis[19]. A patient with type IV lacks cytochrome b_5 and has cyanosis without neurological abnormalities[20]. In this patient the activity of cytochrome b_5 reductase in tissues other than red cells was not assayed. As no factor evident from the history or physical examination could explain the severe neurological deficits in this patient, it was

determined that the neurologic type II form of the hereditary enzymopenic methemoglobinemia was present.

In summary, we have identified a 3-year-old Thai boy with type II hereditary enzymopenic methemoglobinemia. The severity of the phenotype makes prenatal diagnosis justified. Because of the ability to make a definite diagnosis in the proband, a method to perform prenatal diagnosis is now available to the parents.

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เด็กชายไทยซึ่งป่วยด้วยโรค hereditary enzymopenic methemoglobinemia ชนิดที่ 2

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ผู้ป่วยที่มีอาการเขียวอาจเกิดจากการที่มีระดับเมธฮีโมโกลบินมากกว่า 1.5 กรัม/เดซิลิตร ภาวะเมธฮีโมโกลบินนีเมียอาจเกิดจากการถ่ายทอดทางพันธุกรรมซึ่งแบ่งได้เป็น 2 กลุ่ม กลุ่มแรกเกิดจากเฮ็มฮีโมโกลบินซึ่งถ่ายทอดแบบยีนเด่น กลุ่มที่ 2 เกิดจากความผิดปกติของเอ็นไซม์ซึ่งถ่ายทอดแบบยีนด้อย ในกลุ่มหลังนี้ยังแบ่งได้อีกเป็น 4 ชนิด โดยชนิดที่ 2 เกิดจากการขาด cytochrome b_5 reductase ในหลายเนื้อเยื่อรวมทั้งสมอง เป็นผลให้ผู้ป่วยกลุ่มนี้มีอาการทางระบบประสาทที่รุนแรงร่วมด้วย

ในบทความนี้เรารายงานผู้ป่วยเด็กชายไทยอายุ 3 ปีซึ่งเป็นโรคเมธฮีโมโกลบินนีเมียประเภทที่เกิดจากการถ่ายทอดทางพันธุกรรมเนื่องจากการขาดเอ็นซัยม์ชนิดที่ 2 บิดาและมารดาของผู้ป่วยเป็นญาติกัน ผู้ป่วยเริ่มเขียวเมื่ออายุ 10 เดือน ชักเมื่ออายุ 1 ปี มีขนาดศีรษะเล็กและพัฒนาการช้ามากโดยยังไม่สามารถชันคอได้ การตรวจทางระบบหัวใจ หลอดเลือด และระบบทางเดินหายใจไม่พบสิ่งผิดปกติ การตรวจระดับเมธฮีโมโลบินด้วยวิธีวัดการดูดซับแสงพบเมธฮีโมโลบิน 18% การตรวจระดับการทำงานของเอ็นไซม์ด้วยวิธีเชิงคุณภาพยืนยันการขาดเอ็นซัยม์ cytochrome b_5 reductase วิธีการนี้จะสามารถนำมาตรวจเพื่อให้การวินิจฉัยก่อนคลอดกับน้องผู้ป่วยในกรณีที่บิดามารดาต้องการมีบุตรอีก

คำสำคัญ : Methemoglobinemia (เมธฮีโมโกลบินนีเมีย), Cytochrome b_5 Reductase

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บทความที่ 8

A Cost-Benefit of GnRH Stimulation Test in Diagnosis of Central Precocious Puberty (CPP)

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Abstract

The GnRH stimulation test is the gold standard to diagnose central precocious puberty (CPP). Conventionally, we need at least 2 hours to finish the test which seems to be costly and time consuming. In this study, we described the pattern of LH and FSH levels during the GnRH test in 27 girls who presented with various degrees of precocious puberty. We found that the blood samples at 90 and 120 min after GnRH were not necessary. To save the cost of diagnosis, the basal LH/FSH ratio > 0.2, the 30 min LH/FSH ratio after GnRH > 0.9 and the peak LH/FSH ratio > 1.0 can be used to diagnose CPP with positive predictive values (PPV) of 87.3, 89.4 and 93.8 per cent respectively.

Key word : Precocious Puberty

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BACKGROUND

Normal pubertal development in humans requires the activation of the luteinizing hormone releasing hormone (LHRH) pulse generator at the appropriate time, 9-13 years in girls and 10-14 years in boys. During the prepubertal period, the LHRH pulse generator is in the juvenile pause secreting very low levels of gonadotropin. Any conditions affecting the early activation of LHRH pulse generator may cause central or true precocious puberty. However, not all girls presenting with early breast development may have precocious puberty. They may have the benign condition which is called premature thelarche and treatment is not required. A previous study hypothesized that premature thelarche and central precocious puberty may represent different positions along a continuum of hypothalamic LHRH neuron activation[1]. The diagnosis of central precocious puberty (CPP) requires many factors including age of onset, degree of advancement in sexual and skeletal maturation, tempo of

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progression and the standard laboratory confirmation of central precocious puberty which is the maximal serum luteinizing hormone (LH) concentration after gonadotropin-releasing hormone (GnRH) stimulation[2]. Because bone age advancement is usually found in CPP, eventually, resulting in short adult height if treatment does not intervene[3]. The conventional test requires 5 blood samples including the basal sample for LH, FSH and estradiol and subsequently every 30 minutes after 100 microgram of GnRH (Relisorm-L) for LH, FSH at 30, 60, 90 minutes and for LH, FSH and estradiol at 120 minutes. The test seems to be costly and time-consuming.

In this study, we describe the pattern of LHRH pulse generator during GnRH stimulation test in girls presenting with breast development and in those with early breast development and other signs of puberty such as increased height velocity, pubic hair and menstruation. Regarding the cost-benefit of conventional GnRH test, we evaluated the basal LH, FSH levels and LH/FSH ratio to determine whether they could be used instead of the conventional test to confirm CPP.

MATERIAL AND METHOD

All girls who presented with early breast development before 8 years of age were included in this study and divided into 3 groups depending on the severity of precocious puberty. (Table 1)

Group I : Nine girls presented with early breast enlargement and no other signs of puberty. No advancement of bone age and no history of increased height velocity.

Group II : Ten girls presented with early breast enlargement and no other signs of puberty. Bone age advancement at least one year over the chronological age was demonstrated. Some of them also had history of increased height velocity.

Group III : Eight girls presented with early breast development and other signs of puberty such as pubic hair development or menstruation.

GnRH stimulation tests were performed in all girls and FSH, LH were measured at 0, 30, 60, 90, 120 min and estradiol at 0 and 120 min after giving synthetic GnRH (Relisorm 100 ug) intravenously. The bone age was estimated by the Greulich & Pyle method. Pelvic ultrasonography was performed to exclude ovarian tumor or functional ovarian cysts. Tumor markers including hCG and alpha-fetoprotein were also measured.

Serum FSH, LH and estradiol levels were measured by fluoroimmunoassay.

The mean FSH, LH and estradiol levels were compared between the groups and within the group but at different times.

The statistics used in this study were *t* test and ANOVA and $p < 0.5$ was considered significant.

RESULTS

From all 27 GnRH tests, 20/27 (74.1%) had the peak serum LH at 30 min after GnRH, 6/27 (22.2%) at 60 min and 1/27 (3.7%) at 90 min. No one had peak LH at 120 min.

The peak FSH occurred at 30 min in 7 out of 27 (26%), 10/27 (37%) at 60 min, 5/27 (18.5%) at 90 min and 5/27 (18.5%) at 120 min.

In group I, the mean peak LH was 7.1±4.1 IU/L and FSH 13.46 ± 2.7 IU/L. (Table 2)

The basal LH/FSH ratio was 0.07±0.05 and the peak LH/FSH was 0.53 ± 0.34 (Fig. 1, 2).

Table 1. The clinical data of patients in 3 groups.

Group	N	CA (yr)	Breast stage	Pubic hair	Menstruation
I	9	7.4±1.2	2.1±0.3	I	no
II	10	7.8±0.8	2.7±0.5	I	no
III	8	8.8±4.0	3.5±0.8	1.8±0.5	all
Group	**BA (yr)**	**HtSDS**	**HtSDS for BA**	**Wt SDS**	
I	7.4±1.1	0.5±0.9	0.3±0.5	0.5±1.0	
II	10.5±0.7	1.6±0.7	-0.3±0.7	1.5±0.8	
III	11.7±1.5	2.9±1.4	-0.1±1.2	3.7±1.9	

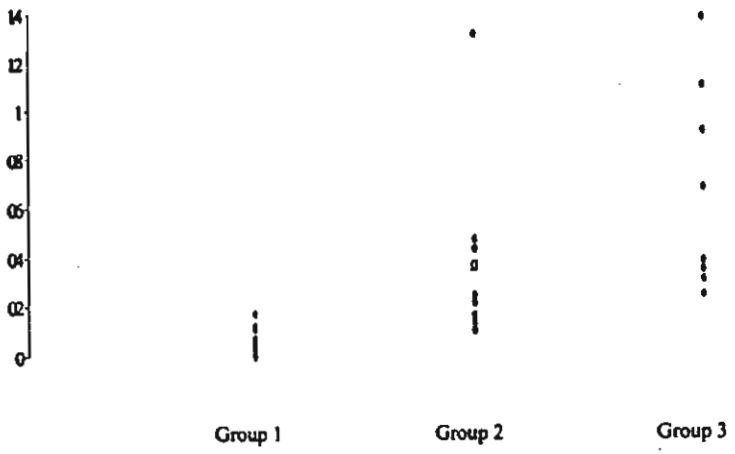

Fig. 1. The basal LH/FSH ratio in 3 groups.

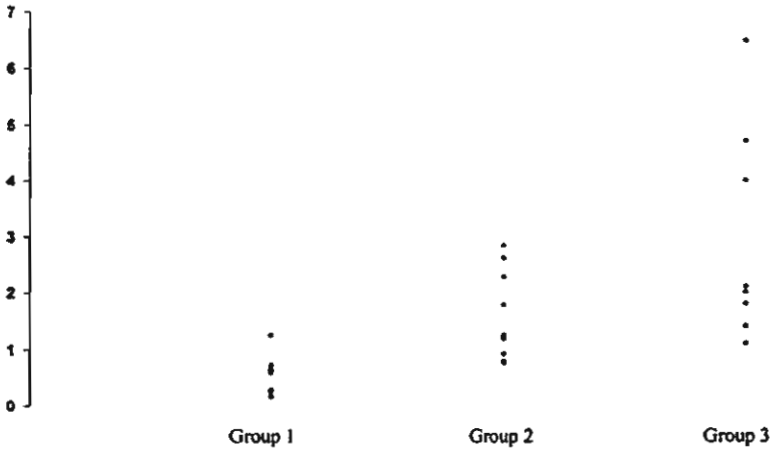

Fig. 2. The peak LH/FSH ratio in 3 groups.

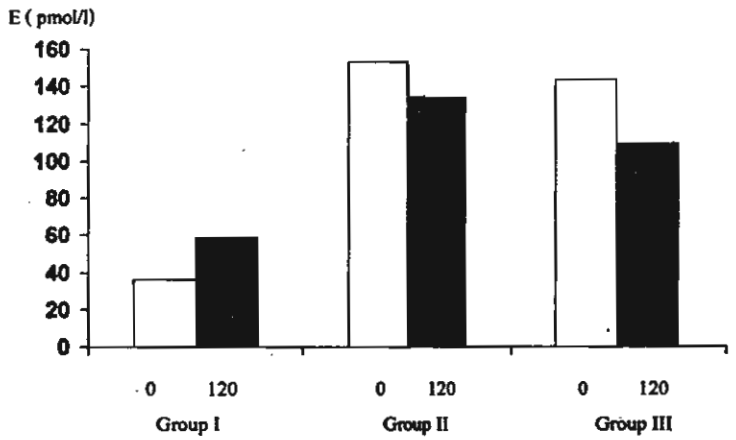

Fig. 3. Estradiol (E) at 0 and 120 min in 3 groups.

Table 2. Serum LH, FSH, LH/FSH, estradiol in 3 groups.

Group	Basal LH (IU/L)	Basal FSH (IU/L)	Peak LH (IU/L)	Peak FSH (IU/L)
I	0.2±0.17	3.98±3.91	7.1±4.1	13.46±2.7
II	1.86±1.45	5.06±1.38	18.75±11.5	12.44±4.76
III	3.65±2.52	5.21±1.85	24.08±13.15	9.31±2.37
Group	**Basal LH/FSH**	**Peak LH/FSH**	**Basal E2 (pmol/l)**	**120 min E2 (pmol/l)**
I	0.07±0.05	0.53±0.34	36.19±22.05	59.21±73.96
II	0.38±0.35	1.57±0.77	153.5±148.5	134.8±113.6
III	0.66±0.41	2.96±1.92	144.2±116.8	110.0±54.1

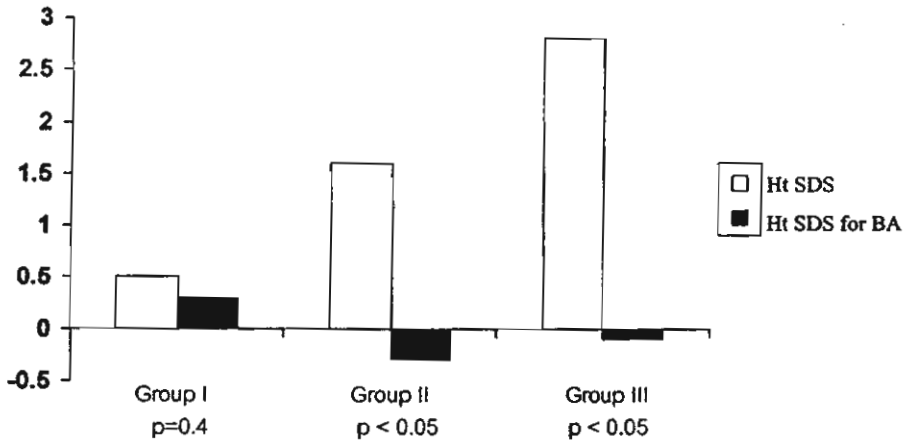

Fig. 4. Ht SDS and Ht SDS for BA in 3 groups.

The means of estradiol at 0 and 120 min were 36.19 ± 22.05 and 59.21 ± 73.96 pmol/l which were not significantly different. (Fig. 3)

In group II, the mean peak LH was 18.75 ± 11.5 IU/L and FSH 12.44 ± 4.76 IU/L (Table 2).

The basal LH/FSH ratio was 0.38 ± 0.35 and the peak LH/FSH 1.57 ± 0.77. (Fig. 1, 2)

The means of estradiol at 0 and 120 min were 153.5 ± 148.5 and 134.8 ± 113.6 pmol/l which were not significantly different. (Fig. 3)

In group III, the mean peak LH was 24.08 ±13.15 IU/L and FSH 9.31 ± 2.37 IU/L. (Table 2)

The basal LH/FSH ratio was 0.66 ± 0.41 and peak LH/FSH 2.96 ± 1.92. (Fig. 1, 2)

The means of estradiol at 0 and 120 min were 144.2 ± 116.8 and 110.0 ± 54.1 pmol/l which were not significantly different. (Fig. 3)

In contrast to the patients in group II and III, the patients in group I had good height prognosis because Ht SDS and Ht SDS for BA were not significantly different. (Fig. 4) If we considered the peak LH > 10 IU/L as the laboratory confirmation of CPP, we found that all patients in group III, 8 of 10 patients in group II and 1 of 9 patients in group I had CPP. Therefore, most of the patients in group I were in the benign group called premature thelarche but most of them in group II and III were in the more serious group (CPP) and treatment should be considered.

The peak LH/FSH ratio of 1.0 may be used to differentiate between premature thelarche and CPP with the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of 88.2, 90, 93.8, 81.8 per cent respectively.

Table 3. The cost and benefit of the tests for diagnosis of CPP.

TEST	Sensitivity (%)	Specificity (%)	Positive predictive value (PPV) (%)	Negative predictive value (NPV) (%)	Cost (baht)*
Basal LH/FSH = 0.2	82.5	80	87.5	72.7	700
Peak LH/FSH = 1.0	88.2	90	93.8	81.8	3400
30 min LH/FSH = 0.9	100	80	89.4	100	1500

* 38 baht ≈ 1 US dollar

Additionally, the basal LH/FSH ratio of 0.2 and the 30 min LH/FSH ratio of 0.9 may also be used. The sensitivity, specificity, PPV, NPP and costs of all tests are shown in Table 3.

From this study, no one had ovarian tumor or cyst producing sex hormone.

One of the patients in group I presented with stage II breast development and no advancement of bone age, however, the test showed the peak LH of 16.4 and the peak FSH of 13.2 IU/L, The basal LH/FSH ratio of 0.12 and peak LH/FSH ratio of 1.24 and the 30 min LH/FSH ratio of 1.4. On follow-up, we found that her puberty had progressed and LHRH analogue was started subsequently.

All patients in group III having clinical grounds and laboratory confirmation of CPP met the three cut-off points to diagnose precocious puberty, basal LH/FSH>0.2, peak LH/FSH >1 and 30 min LH/FSH > 0.9.

However, 2 of 10 in group II had peak LH < 10 (8 and 9.5) but clinical grounds supported precocious puberty such as advancement of bone age, increased height velocity and treatment was considered because of the progression of puberty. We found that the first girl had all 3 cut-off levels (basal LH/FSH 0.47, peak LH/FSH 1.9 and 30 min LH/FSH 1.2) and the second girl had 2 out of 3 cut-off levels. (basal LH/FSH 0.37, peak LH/FSH 0.92, 30 min LH/FSH 1)

The weight SDS was higher in group II and III than in group I.

DISCUSSION

The available gold standard used at present to diagnose central precocious puberty (CPP) is the LH-predominant response to GnRH stimulation test.[2,4] Neely et al suggested that the peak LH > 5 IU/L after GnRH stimulation test considered CPP because this figure was above +2 SD for normal prepubertal female subjects[5,7]. However, some studies recommended different figures e.g. > 8 or maximum night time LH > 10 IU/L[6-8]. The peak FSH after GnRH cannot be used to diagnose CPP. From this study, the peak FSH levels in the 3 groups were not significantly different but the changes were seen in the peak LH levels which increased progressively from group I to group III. (Fig. 5) This finding represented the maturation of the LHRH pulse generator of which the LH levels

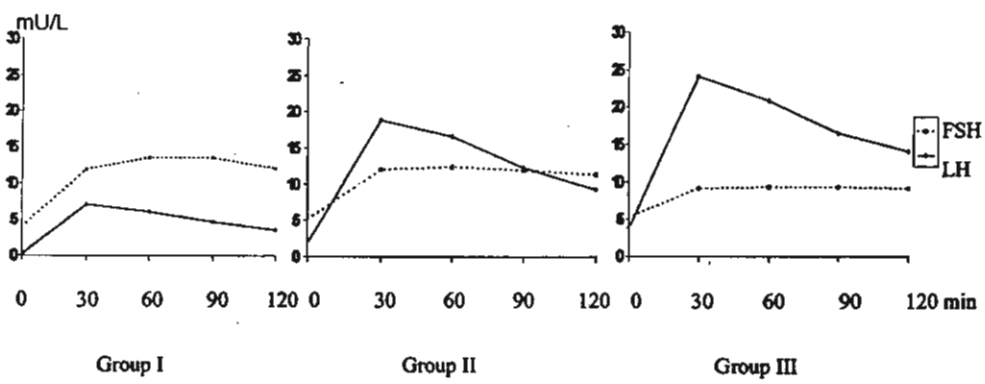

Fig. 5. FSH and LH during GnRH test in 3 groups.

but not FSH had progressively increased from prepubertal to pubertal period. Most of the peak LH levels occurred at 30 min after GnRH and almost 100 per cent occurred at 60 min. The peak FSH occurred at 30, 60, 90, 120 min for 26 per cent, 37 per cent, 18.5 per cent and 18.5 per cent respectively. However, the levels at 4 different times were not significantly different. In addition, the mean E2 at 120 min was not different from the basal E2. To save costs, therefore, we suggested that it was not necessary to take a sample at 90 and 120 min during the GnRH test.

Previous study showed that the spontaneous LH levels correlated strongly with the peak LH after GnRH and it was recommended to use the spontaneous LH to diagnose CPP. The spontaneous level LH > 0.1 mU/L by ICMA detected CPP with 94 per cent sensitivity and 88 per cent specificity (4). Similar results were demonstrated in many studies(8-10). The different immunometric assays with simple multiplication factors were inaccurate(11). Therefore, the peak LH/FSH ratio may be the best predictor for CPP. From our study, if we used the peak LH/FSH, we would reach better sensitivity, specificity and PPV than using the basal LH/FSH. Similar to the study by Oretor et al which suggested that the peak LH/FSH ratio was the best predictor for CPP(7). Angsusingha et al also suggested that the peak LH minus basal LH was the best parameter to diagnose CPP(13). The cost of the standard GnRH test is very expensive and takes at least 2 hours to finish the test. Therefore, we may use the blood sample at 30 minutes after GnRH intravenous which is cheaper, saves time and can be done in out-patient clinics to diagnose CPP and the results are not apparently different. As in a previous study(9), the single sample subcutaneous GnRH test can be used to confirm CPP. Even the basal LH/FSH ratio which is the cheapest way to diagnose CPP can be used in conjunction with clinical ground to diagnose with PPV of 87.5 per cent (Table 3).

The increased adipose tissue was proved to be associated with early puberty in girls(12). In the present study, we supported this because the wt SDS was higher in group II and III than in group I.

The decision to start treatment in girls with early breast development relies not only on the biochemical evidence, but we also have to consider the clinical data of each individual. The biochemical result is a good tool to confirm CPP but it should not be too expensive and should be easy to perform. Furthermore, clinical follow-up is very important to make the decision for treatment in patients with CPP.

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การประเมินการใช้ GnRH stimulation test ในการวินิจฉัยภาวะ Central Precocious Puberty

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การวินิจฉัยภาวะ central precocious puberty (CPP) ซึ่งเป็นที่ยอมรับกันโดยทั่วไป จำเป็นต้องทำ GnRH stimulation test การทำ test ดังกล่าวต้องใช้เวลาประมาณ 2 ชั่วโมงซึ่งทำให้เสียเวลาและค่าใช้จ่ายมาก คณะผู้วิจัยได้ทำการศึกษารูปแบบของระดับ LH และ FSH ระหว่างการทำ GnRH test ในเด็กผู้หญิงจำนวน 27 ราย ที่มาพบด้วยเรื่องเป็นสาวก่อนวัยอันควรที่รุนแรงแตกต่างกัน และพบว่าในการวินิจฉัยดังกล่าว ไม่จำเป็นต้องทำการตรวจเลือดที่เวลา 90 และ 120 นาที นอกจากนั้นการใช้ระดับ basal LH/FSH ที่มีค่ามากกว่า 0.2, LH/FSH ที่ 30 นาที หลังให้ GnRH มากกว่า 0.9 และ peak LH/FSH ที่มีค่ามากกว่า 1.0 สามารถทำนายภาวะ CPP ได้ถูกต้องเท่ากับ ร้อยละ 87.3, 89.4 และ 93.8 ตามลำดับ

คำสำคัญ : หนุ่มสาวก่อนวัยอันควร

สุทธิพงศ์ วัชรสินธุ และคณะ
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บทความที่ 9

CLINICAL AND MOLECULAR CHARACTERISTICS OF THAI PATIENTS WITH ACHONDROPLASIA

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Abstract. Achondroplasia is an autosomal dominant disorder characterized by disproportionately short stature, frontal bossing, rhizomelia, and trident hands. Most patients appear sporadically resulting from a *de novo* mutation associated with advanced paternal age. A glycine to arginine mutation at codon 380 (G380R) of the fibroblast growth factor receptor 3 gene (*FGFR3*) was found to be the most common cause of achondroplasia in various populations. We identified and clinically characterized 3 Thai patients with achondroplasia. In all of them, we also successfully identified the G380R mutation supporting the observation that this is the most common mutation in achondroplasia across different ethnic groups including Thai.

INTRODUCTION

Patients with short stature display an extremely long list of differential diagnoses. Achondroplasia is one of them. Clinical manifestations and molecular defects of patients with achondroplasia have been described in various ethnic groups. Here we report three Thai patients with achondroplasia whose molecular abnormalities were successfully identified, providing a specific method for molecular diagnosis of patients and for prenatal diagnosis in families at risk.

MATERIALS AND METHODS

Case reports: Three patients coming to the Genetics Clinic at King Chulalongkorn Memorial Hospital were diagnosed with achondroplasia. Patient 1 was born at term to a 37 year-old G3P2 Thai mother and a 43 year-old unrelated Thai father. Neither the parents nor the two elder sisters of patient 1 were affected. Pregnancy and delivery were uncomplicated. His birth weight was 3,590 g (+1 SD), length 47 cm (-2 SD), and head circumference 38.5 cm (+3 SD). In addition to short stature, physical examination revealed increased upper to lower trunk ratio (2.2:1) (normal 1.7:1), frontal bossing, rhizomelia, trident hands, left hydrocele, and lordosis (Fig 1A). Achondroplasia was diagnosed soon after birth. At 8 months of age, his head circumference was 49 cm (+4 SD). Due to the rapid increase of his head size, a CT scan of the brain was performed revealing hydrocephalus. A ventriculoperitoneal shunt was placed. Developmental assessment by the Gesell Developmental schedule showed a developmental quotient of 73 at the chronological age of 1 year and 8 months. The left hydrocele was surgically repaired at 1 year and 9 months. Polysomnography performed at 2 years and 6 months was normal. At 4 years and 6 months, growth hormone provocative tests by insulin and clonidine showed maximum growth hormone levels of 1.9 and 6.4 ng/ml, respectively, indicating growth hormone deficiency. The IQ test by WISC III revealed verbal IQ, performance IQ and full IQ of 84, 103, 93 respectively at 8 years of age. Radiography of the lumbar spine showed caudal narrowing

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of the spinal canal with short pedicles (Fig 2A). At his last follow-up at 8 years and 1 month, his height was 100.2 cm (-4 SD), weight 19.6 kg (-1 SD), and head circumference 56 cm (+2.5 SD).

Patient 2 was born at term to a 27-year-old G1P0 Thai mother and a 27-year-old unrelated Thai father. The parents were unaffected. Pregnancy, labor and delivery were unremarkable. His birth weight was 3,500 g and his length 47 cm. Physical examination at 4 months of age revealed macrocephaly with a head circumference of 43 cm (+2 SD), increased upper to lower trunk ratio (40:19.5 = 2.05:1), large anterior fontanel, frontal bossing, depressed nasal bridge, trident hands, and rhizomelia (Fig 1B). Radiography revealed decreased interpeduncular distances of his lumbar vertebrae. A diagnosis of achondroplasia was made. CT scan of the brain at 10 months revealed hydrocephalus requiring ventriculoperitoneal shunt. Developmental assessment by the Gesell Developmental schedule showed a mental age of 39 weeks at the chronological age of 79 weeks. The IQ test according to Stanford Binet revealed an IQ of 82 at 5 years of age. Echocardiogram performed at 2 years and an eye examination at 3 years were unremarkable. Noisy breathing was developed at the age of 5 years. Obstructive sleep apnea was found by polysomnography and his hypertrophied tonsils and adenoids were removed at the age of 5 years and 10 months. The following tests were normal: blood cell counts, blood sugar, BUN, Cr, electrolytes, prothrombin time, and partial thromboplastin time. At his last visit at the age of 6 years and 10 months his height was 99.3 cm (-2.5 SD), weight 31.4 kg (+2.5 SD), and head circumference 54 cm (+1.5 SD).

Patient 3 was born at term after uncom-

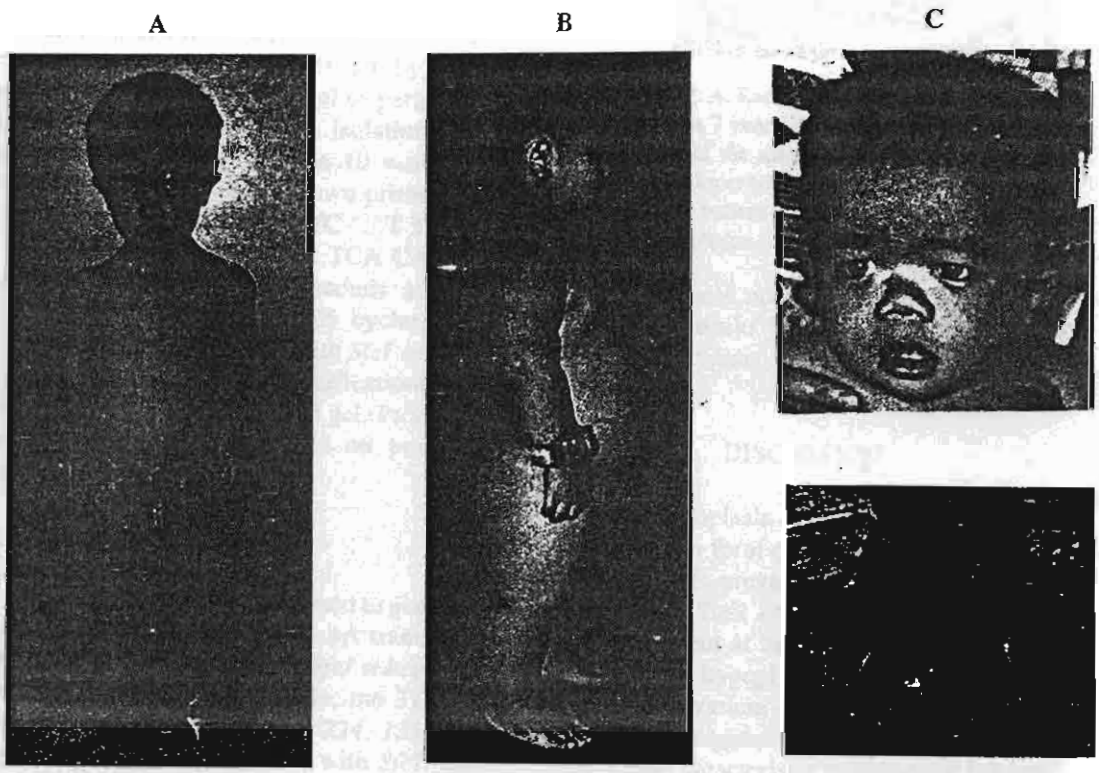

Fig 1–Clinical features. A. Patient 1 at 7 years of age showing disproportionate short stature with rhizomelia. B. Patient 2 at 6 years old revealing frontal bossing, overweight, and lumbar lordosis. C. Patient 3 at 11 months old showing maxillary hypoplasia (upper panel) and a trident hand (lower panel).

plicated pregnancy and delivery to a 36-year-old G3P2 Thai mother and a 39-year-old unrelated Thai father. Parents and the two elder siblings of patient 3 were unaffected. His birth weight was 3,400 g. Physical examination at the age of 11 months showed his weight at 6,900 g (-2.5 SD), length 62 cm (-5 SD), head circumference 47.5 cm (+2 SD), and arm span 58 cm. He had frontal bossing, midface hypoplasia, trident hands, kyphosis, and rhizomelia (Fig 1C). Developmentally, at 1 year of age, he could not sit unsupported but was able to do pincer grasp and talked a few words. Radiography of the spine revealed dextroscoliosis and narrowing of the interpeduncular distance of the lumbar vertebrae. Echocardiogram performed at 1 year of age was normal. CT scan of the brain at 1 year of age showed communicating hydrocephalus requiring lumboperitoneal shunt placement (Fig 2B).

Mutation analysis

After informed consent was obtained in accordance with the standards set by local institutional review boards, 6 ml of peripheral blood were obtained for DNA isolation by a standard method. *FGFR3* exon 10 was PCR amplified using the following two primers: 5′ CTC TGG GCC AGG GGA ATC CAT 3′ and 5′ GGCTGC AGA GAG GGC TCA CAC 3′. The PCR conditions were 30 seconds at 94°C and 90 seconds at 68°C for 35 cycles. The PCR products were digested with *SfcI* according to the manufacturer's specifications and electrophoresed on a 2% agarose gel (Promega) stained with ethidium bromide on preparation.

RESULTS

The PCR amplification was used to generate a 372 bp fragment. The 1138G→A transition of the *FGFR3* gene creates an *SfcI* restriction site. Hence, in the mutant allele, the 372 bp product is cleaved by *SfcI* into 234, 131 and 7 bp fragments. After digestion with *SfcI*, the PCR products of all three patients yielded 3 bands of 372, 234 and 131 bp. The expected

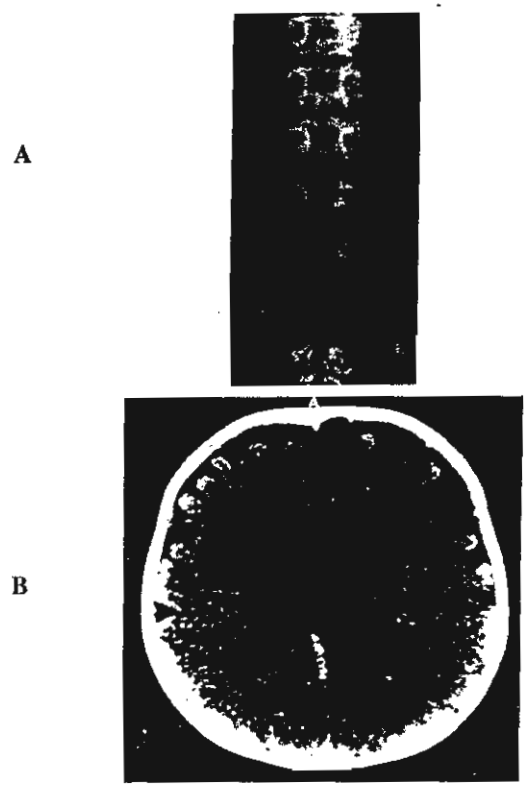

Fig 2–Imaging. A. Radiograph of lumbar spine of patient 1 at 7 years of age revealing caudal narrowing of the spinal canal and a shadow of a ventriculoperitoneal shunt. B. CT scan of the brain of patient 3 at 11 months old revealing hydrocephalus.

7 bp band could not be seen due to its small size. These results indicated that all of them were heterozygous for the 1138G→A transition.

DISCUSSION

Achondroplasia (MIM 100800), is the most common form of short-limbed dwarfism in humans. Its prevalence is estimated to be 1 in 20,000 (Stoll *et al*, 1989). The physical features evident at birth include frontal bossing, midface hypoplasia, rhizomelia, trident hands, genu varum, limitation of elbow extension, and exaggerated lumbar lordosis (Hall, 1992). The characteristic radiological features include caudal narrowing of the interpedicular distance (Oberklaid *et al*, 1979). We found

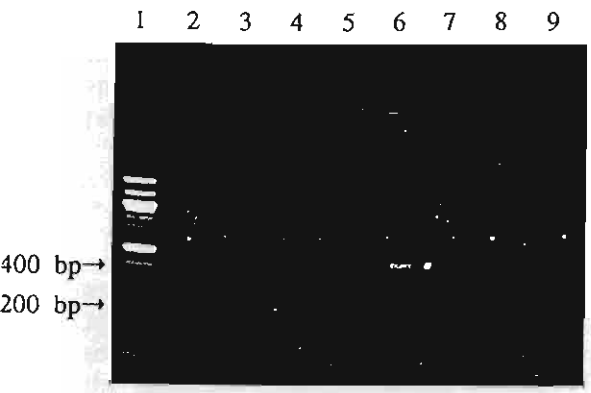

Fig 3–Restriction enzyme detection of the G380R mutation in achondroplasia. Lane 1 represents a 100 bp marker with the bands at 200 and 400 bp indicated with arrows. Lanes 2 and 3 were of the mother of patient 1; lanes 4 and 5 patient 1; lanes 6 and 7 patient 3; lanes 8 and 9 the mother of patient 3. Lanes 2, 4, 6 and 8 were PCR products without adding restriction enzymes and only the undigested 372 bp bands were presented. Lanes 3, 5, 7, and 9 were PCR products mixed with *SfcI*. The new bands of 231 bp and 134 bp in lanes 5 and 7 demonstrate that these individuals are heterozygous for the 1138G→A mutation. The products of their mothers in lane 3 and 9 were not cleaved by *SfcI*, which serves as negative controls.

3 patients with features typical for achondroplasia. In addition, they all have hydrocephalus requiring shunt placement to decrease the intracranial pressure. Ventriculomegaly in achondroplastic children was shown to accompany hydrocephalus, which is likely secondary to increased intracranial venous pressure due to hemodynamically significant stenosis of the jugular foramen and jugular venous obstruction at the level of the thoracic inlet (Steinbok *et al*, 1989). Patient 2 also had obesity. Obesity has been shown to be a significant health problem in achondroplasia (Hecht *et al*, 1988). Weight should be closely monitored and dietary intervention instituted whenever patients are overweight (American Academy of Pediatrics Committee on Genetics, 1995). All of our patients displayed noisy breathing, which is one of the known complications in achondroplasia (Stokes *et al*, 1983). Although delayed in early motor development, all of our patients showed intelligence within the normal range, consistent with most achondroplastia patients (Brinkmann *et al*, 1993).

Genetically, achondroplasia is inherited in an autosomal dominant fashion with complete penetrance (Tanaka, 1997). Eighty to 90% of cases are sporadic and associated with advanced paternal age (Stoll *et al*, 1989). After the gene had been cloned, molecular work has confirmed that mutations of the *FGFR3* gene in sporadic cases of achondroplasia occur exclusively on the paternally derived chromosome (Wilkin *et al*, 1998). All of our three achondroplastia patients are sporadic cases. The paternal ages of patients 1 and 3 were advanced (43 and 39 years).

Molecularly, the gene responsible for achondroplasia has been mapped to chromosome 4p16.3 (Velinov *et al*, 1994; Le Merrer *et al*, 1994; Francomano *et al*, 1994). Shortly after the gene had been mapped, the mutation of the fibroblast growth factor receptor-3 (*FGFR3*) gene was identified (Shiang *et al*, 1994; Rousseau *et al*, 1994). More than 99% of achondroplasia is caused by an *FGFR3* G380R mutation. Bellus *et al* (1995) found that 150 out of 154 unrelated patients showed the 1138G→A transition and 3 the 1138G→C transversion. Achondroplasia patients of other ethnic groups including Swedes, Chinese, Japanese, Jews and Arabs also have the most common mutations resulting in the G380R (Alderborn *et al*, 1996; Niu *et al*, 1996; Tanaka, 1997; Passos-Bueno *et al*, 1999; Katsumata *et al*, 2000; Falik-Zaccai *et al*, 2000). This study revealed that Thai achondroplasts also had the 1138G→A transition resulting in G380R as the most common mutation. Even though the patients are all sporadic reducing the recurrence risk to far below 50% in younger siblings of the patients, the risk is not negligible. Owing to advanced molecular techniques, a powerful method to perform prenatal diagnosis is now available to the parents.

In summary, we have identified three unrelated Thai patients with achondroplasia. They all display the 1138G→A mutation of the *FGFR3* gene supporting the observation

that this is the most common mutation responsible for the phenotype across different populations.

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บทความที่ 10

A CASE OF PFEIFFER SYNDROME TYPE 1 WITH AN A344P MUTATION IN THE *FGFR2* GENE

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Abstract. Pfeiffer syndrome, an autosomal dominant disorder, consists of craniosynostosis, broadening of the thumbs and great toes, and partial soft tissue syndactyly of the hands and feet. Three clinical subtypes have been classified mainly for the purpose of genetic counseling. Mutations in *FGFR1* and *FGFR2* are known to be associated with the syndrome. However, the correlation between genotype and phenotype is not well defined. Only one patient with Pfeiffer syndrome with no other clinical information has been reported to have had an A344P mutation of the *FGFR2*. Here we report a Thai male patient with sporadic Pfeiffer syndrome type 1 with impaired intelligence (IQ = 77). Mutation analysis revealed A344P in *FGFR2*. Identification of the clinical features and molecular defects in more patients is required to better correlate the genotype and phenotype of this complex syndrome.

INTRODUCTION

Pfeiffer syndrome (MIM 101600), an autosomal dominant disorder, consists of craniosynostosis, broadening of the thumbs and great toes, and partial soft tissue syndactyly of the hands and feet (Martsolf *et al*, 1971). Three clinical subtypes have been delineated by Cohen (1993). Patients with type 1 have the classical phenotype with normal to near normal intelligence. Affected individuals with type 2 have a cloverleaf skull, severe CNS involvement, and do poorly with early death. Type 3 is similar to type 2 with the absence of a cloverleaf skull. Most cases of Pfeiffer syndrome are sporadic. In familial cases, autosomal dominant inheritance with complete penetrance is characteristic. Variable expressivity has involved mostly the presence and the degree of syndactyly (Cohen, 1993).

On the molecular level, Pfeiffer syndrome displays locus heterogeneity. The first gene identified to be responsible for the syndrome was *FGFR1* (MIM 136350) (Muenke *et al*, 1994). A year later, a second locus, *FGFR2* (MIM 176943), was found (Schell *et al*, 1995). At least 24 different mutations in *FGFR2* associated with the Pfeiffer phenotype have been characterized (Passos-Bueno *et al*, 1999). Some mutations have been reported to cause a specific clinical type such as Ser351Cys which was found in a patient with Pfeiffer syndrome type 3 (Gripp *et al*, 1998). Only one patient with Pfeiffer syndrome has been reported to have an A344P mutation of the *FGFR2* (Meyers *et al*, 1996). No other information about this patient was given. Identification of the clinical features and molecular defects in more patients is required to better correlate the genotype and phenotype of this complex syndrome.

Here we report a Thai male patient with sporadic Pfeiffer syndrome type 1 with impaired intelligence. Mutation analysis revealed A344P in *FGFR2*. He represents the second

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case of this mutation reported to date.

MATERIALS AND METHODS

Case report

A male patient was born at term to a 41-year-old G2P1 Thai mother and a 40-year-old unrelated Thai father. Prenatal history was unremarkable. A cesarean section was performed because of premature rupture of membranes. Birth weight was 3400 g (75th centile), birth length 51 cm (75th centile), and head circumference 37 cm (>90th centile). Physical examination at 4 months of age revealed bicoronal synostosis, proptosis, midface hypoplasia, micrognathia, and enlarged great toes. A diagnosis of Pfeiffer syndrome was given. The patient underwent frontoorbital advancement at 7 months old. The patient had obstructive sleep apnea requiring adenoidectomy and uvuloplasty at 1½ years old. His IQ at 3 years and 2 months old was 77. His last clinic visit was at 6 years of age (Fig 1). At this time his height was 108 cm (25th centile), his weight 15 kg (10th centile), and his head circumference 50.5 cm (between 10th and 25th centile). Turribrachycephaly, protosis, and midface hypoplasia were noted. His thumbs were slightly broadened. The great toe/second toe ratios were 1.96 on the right and 1.74 on the left. The physical features of his parents and brother revealed no major malformations.

Mutation analysis

After informed consent was obtained in accordance with the standards set by local institutional review boards, six ml of peripheral blood was obtained for DNA isolation by a standard method. *FGFR1* exon 5, *FGFR2* exon 8, and *FGFR2* exon 10 were PCR amplified. Primers, annealing temperatures and PCR product sizes are shown in Table 1. The PCR products were electrophoresed on a 2% agarose gel (Promega) and stained with ethidium bromide. The visualized band was extracted and purified with a kit (Bio 101), and sequenced in both directions by using an automated DNA sequencer (ABI Prism 310 Genetic

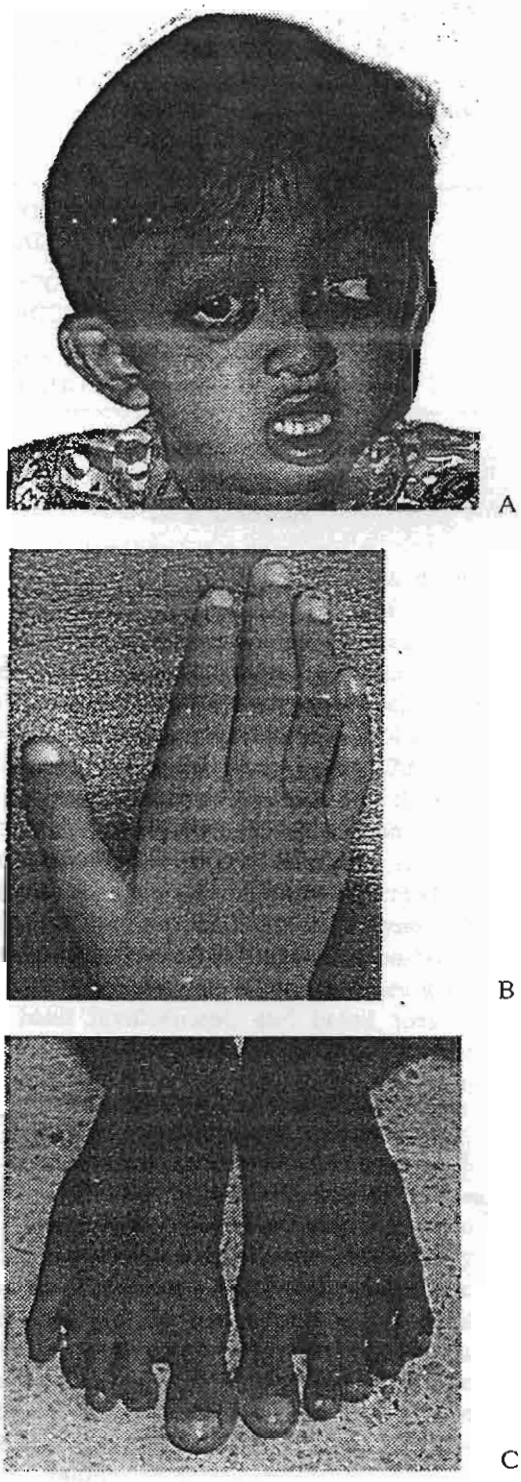

Fig 1–A. Face B. Right hand C. Feet of the patient.

Table 1
Primers, the optimal annealing temperatures, and the PCR product sizes of the exons of the FGFR genes studied.

Gene-Exon	Primers	Annealing temperature	product size
FGFR1-Exon 5	5′-GGAATTCCATCTTCCACAGAGCGG-3′ and 5′- GGAATTCCTCAAGATCTGGACATAAGGCAG-3′	60	216
FGFR2-Exon 8	5′-GGTAGTGGTCTGTCATTCTCCCATC-3′ and 5′-AATCAAAGAACCTGTGGCCAAACCC-3′	60	322
FGFR2-Exon 10	5′-AGCCCCCTCCACAATCATTCCTG-3′ and 5′-TAAAAGGGGCCATTTCTGATAACAG-3′	60	303

Analyser, Perkin Elmer).

RESULTS

A G->C transversion at nucleotide 1209 of the *FGFR2* gene exon 10 was detected (Fig 2). This change substitutes a proline for an alanine residue at amino acid position 344. Sequence tracings of both directions confirmed the mutation. Nucleotide sequences of the *FGFR1* exon 1 and the *FGFR2* exon 8 were normal (data not shown).

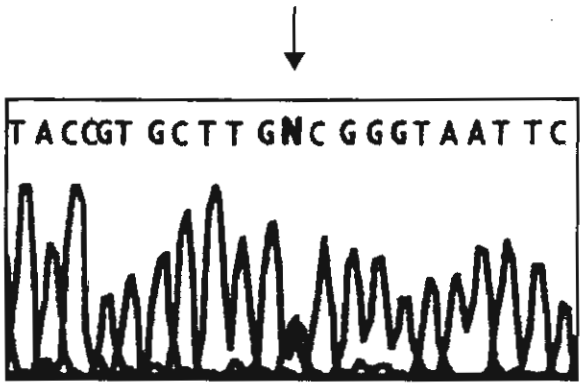

Fig 2–The backward strand sequence of the *FGFR2* exon 10 revealed a G->C transversion (indicated in the figure by an arrow).

DISCUSSION

This patient had craniosynostosis, downslanting palpebral fissures, proptosis and broadening of the thumbs and great toes consistent with Pfeiffer syndrome (Cohen, 1995). The ratios of his hallucal width to second toe width were 1.96 on the right and 1.74 on the left. These are within the range (1.72-2.23) of patients with Pfeiffer syndrome (Cohen, 1993). Although the patient did not have deviation of the thumbs and great toes or syndactyly, these features are not essential for diagnosis. Patients with Crouzon syndrome have normal hands and feet, Jackson-Weiss syndrome is defined by foot anomalies without hand involvement, and broad toes in Saethre-Chotzen syndrome are in the valgus position. Thus, these syndromes may be distinguished from Pfieffer.

Our patient's features are consistent with Pfeiffer syndrome type 1. However, his intelligence seems to be more severely affected by the disease than others. No other family members had similar clinical features. *De novo* mutation is the most likely explaination. His father was 40 years old at the time the patient was born. Advanced paternal age is known to be the risk of *de novo* mutation with the average paternal age of 34.5 ± 7.65 years (Glaser *et al*, 2000).

Molecular study revealed an A344P mutation in *FGFR2* making him the second

case of Pfeiffer syndrome with this mutation. Comparison of the phenotypes between the two patients is not feasible due to no clinical data being available for the first case. Participation with clinical and molecular geneticists in phenotype-genotype studies is necessary to provide more accurate information for genetic counseling.

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บทความที่ 11

A *RET* C634R Mutation in a Thai Female with Multiple Endocrine Neoplasia Type 2A

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Abstract

Multiple endocrine neoplasia type 2A (MEN 2A) is an autosomal dominant disorder characterized by medullary thyroid carcinoma, pheochromocytoma and primary hyperparathyroidism. The first tumor is usually a medullary thyroid carcinoma. MEN 2A is caused by mutations in the *RET* proto-oncogene. The detection of mutations in the gene has important diagnostic and therapeutic impacts. Genetic testing of at-risk family members allows one to identify individuals carrying the mutant alleles with very high specificity and sensitivity. Subsequently, total thyroidectomy, recommended at 5 years of age, can be performed in a prophylactic attempt.

The authors performed a molecular analysis to identify a mutation in a Thai woman with MEN 2A. She was found to be heterozygous for 1900T>C (C634R). The patient had two daughters who were not found to carry the mutation.

The newly available genetic test for patients with MEN 2A in Thailand makes possible accurate DNA-based diagnosis of their at-risk family members before development of the disease, which has important therapeutic impacts for them.

Key word : Multiple Endocrine Neoplasia, RET, Mutation Analysis

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Multiple endocrine neoplasia (MEN) is a hereditary cancer syndrome characterized by the occurrence of tumors involving two or more endocrine glands within a single patient. Two major forms of MEN are recognized and referred to as type 1 (MEN1) and type 2 (MEN2A and MEN2B)[1]. MEN 2A is an autosomal dominant disorder characterized by medullary thyroid carcinoma, pheochromocytoma and primary hyperparathyroidism (OMIM no. 171400). Patients have been reported worldwide including Asia [2-4] and Thailand[5,6]. Germ-line mutations of the *RET* proto-oncogene have been identified as the underlying cause of the disorder[7]. The gene was mapped to chromosome 10q11.2, has 21 exons, and encodes a receptor tyrosine kinase that is expressed in derivatives of neural crest cells. A molecular diagnosis of patients with MEN 2A makes DNA testing of at -risk family members available. It, unlike biochemical tests, permits the unambiguous identification of MEN 2A gene carriers[8]. The identification of a mutation has important implications for clinical management, including lifesaving prophylactic treatment. The authors performed a molecular genetic test to identify a mutation in *RET* in a Thai woman with MEN 2A. This is the first published genetic analysis of MEN 2A in Thailand.

MATERIAL AND METHOD

Case report

A 42-year-old Thai woman was referred to the King Chulalongkorn Memorial Hospital for management of congestive heart failure, uncontrolled hypertension, severe hyperglycemia and bilateral adrenal masses. Details of the patient was previously published[5]. In summary, she was found to have bilateral pheochromocytoma, (Fig. 3) primary hyperparathyroidism and medullary thyroid carcinoma. She underwent bilateral adrenalectomy and subsequently, total thyroidectomy and parathyroidectomy. Her 24-hour urinary metanephrines post-operatively returned to normal range. Her blood pressure and glucose level have been under control with minimal medications. She has two daughters, aged 13 and 6 years.

Mutation analysis

After informed consent was obtained, DNA was extracted from the patient and her two daugthers by a standard method. *RET* exon 11 was polymerase chain reaction (PCR) amplified using 4 µl of gDNA, 1XPCR buffer (Promega, Wisconsin, USA), 1.5 mM $MgCl_2$, 200 µM dNTPs, 0.25 µM of each primer, and 0.4 U Taq DNA polymerase in a total volume of 20 µl. The primer sequences were 5'- GCCATGAGGC AGAGCATA-3' (RET11F) and 5' TGGGGAGGCCA GGGGATCTT-3' (RET11R), yielding a 384-bp product. An initial denaturation step of 94°C for 5 min was followed by 40 PCR cycles, each with a denaturation step of 94°C for 45 s, an annealing of 60°C for 45 s, and an extension of 72°C for 45 s. Amplification cycles were followed by an elongation step of 72°C for 10 min.

PCR products were cloned using pGEM®-T Easy Vector System I (Promega, Wisconsin, USA), according to the manufacturer's recommendations. The PCR products and two plasmid inserts were then sent for sequencing at the National Science and Technology Development Agency, Bangkok, Thailand.

The mutation was confirmed by cleavage of the PCR product with *Hha* I restriction endonuclease (New England BioLabs, Beverly, MA, USA). Twelve µl of PCR product was incubated with the enzyme for 16 h at 37°C.

RESULTS

A heterozygous T>C transition at nucleotide 1900 was identified in *RET* exon 11 of the patient from direct sequencing of the PCR product. One of the

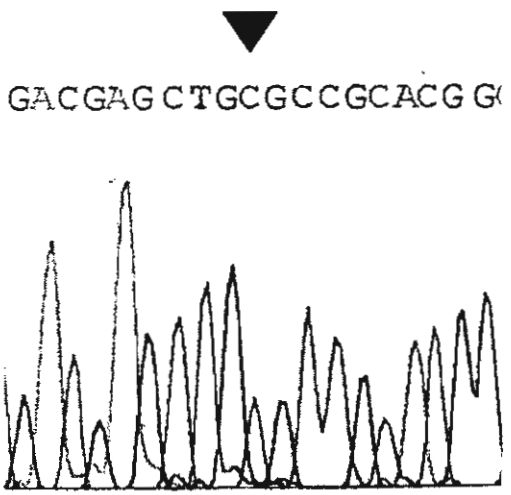

Fig. 1. The sense sequence electropherograms of the plasmid insert of the proband. The arrow head indicates the substitution of a C for the normal T.