รายงานวิจัยฉบับสมบูรณ์

ความหลากหลายของลักษณะทางคลินิกที่เกิดจากการกลายพันธุ์ของยีน FGFR: จากการเติบโตผิดรูปร่างถึงการเกิดมะเร็ง

รองศาสตราจารย์นายแพทย์วรศักดิ์ โชติเลอศักดิ์
หน่วยเวชพันธุศาสตร์และเมแทบอลิซึม ภาควิชากุมารเวชศาสตร์
คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

30 พฤศจิกายน 2546

กิตติกรรมประกาศ

ผู้วิจัยขอขอบพระคุณรองศาสตราจารย์แพทย์หญิงสุมาลี ศรีวัฒนา ศาสตราจารย์นายแพทย์สุทธิพงศ์ วัชรสินธุ รองศาสตราจารย์แพทย์หญิงจรุงจิตร์ งามไพบูลย์ ผู้ช่วยศาสตราจารย์แพทย์หญิงพรรณทิพา ฉัตรชาตรี ผู้ช่วยศาสตราจารย์นายแพทย์ทายาท ตีสุดจิต แพทย์หญิงอวยพร ชีวถาวร แห่งภาควิชากุมารเวชศาสตร์ รองศาสตราจารย์นายแพทย์ จรัญ มหาทุมะรัตน์ ผู้ช่วยศาสตราจารย์นายแพทย์นนท์ โรจน์วชิรนนท์ อาจารย์นายแพทย์พิชิต ศิริวรรณ อาจารย์นายแพทย์อภิชัย อังสพัทธ์ อาจารย์นายแพทย์ ช่อเพียว เดโชพาร แห่งภาควิชาศัลยศาสตร์ ศาสตราจารย์แพทย์หญิงนิยา สุวรรณเวลา ผู้ช่วยศาสตราจารย์แพทย์หญิงกาญจนา โชติเลอศักดิ์ ผู้ช่วยศาสตราจารย์แพทย์หญิงสุกัลยา เลิศล้ำ ผู้ช่วยศาสตราจารย์แพทย์หญิงอัจฉรา มหายศนันท์ ผู้ช่วยศาสตราจารย์แพทย์หญิงปานฤทัย ตรีนวรัตน์ แห่งภาควิชารังสีวิทยา ผู้ช่วยศาสตราจารย์นายแพทย์ วรพงศ์ ภู่พงศ์ ผู้ช่วยศาสตราจารย์แพทย์หญิงสุรางค์ ตรีรัตนชาติ รองศาสตราจารย์นายแพทย์สมชัย นิรุตติศาสน์ แห่งภาควิชาสูตินรีเวชศาสตร์ ผู้ช่วยศาสตราจารย์นายแพทย์สารัช สุนทรโยธิน แห่งภาควิชาอายุรศาสตร์ ผู้ช่วยศาสตราจารย์นายแพทย์ชนพ ช่วงโชติ รองศาสตราจารย์แพทย์หญิงวรนุช ธนากิจ แห่งภาควิชาพยาธิวิทยา คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย นายแพทย์วีรยุทธ ประพันธ์พจน์ โรงพยาบาลราชานุกูล และเจ้าหน้าที่โรงพยาบาลจุฬาลงกรณ์ที่กรุณาร่วมดูแลผู้ป่วยเป็นอย่างดี

ขอขอบพระคุณศาสตราจารย์นายแพทย์ ยง ภู่วรวรรณ รองศาสตราจารย์นายแพทย์อภิวัฒน์ มุทิรางกูร ศาสตราจารย์ปิยะรัตน์ โคสุโขวงศ์ แห่งจุฬาลงกรณ์มหาวิทยาลัย ศ.ดร.มรว. ชิษณุสรร สวัสดิวัตน์ ดร. จันทรกานต์ พิภพมงคล Dr. James R. Ketudat Cairns แห่งสถาบันวิจัยจุฬาภรณ์ Dr. K Yoshiura Dr. N Niikawa แห่ง Nagasaki University School of Medicine, Japan ที่กรุณาให้คำแนะนำ ชี้แนะ สนับสนุนและร่วมทำการทดลองทางห้องปฏิบัติการ

ขอขอบคุณนายชูพงศ์ อิทธิวุฒิ นายเฉลิมพล ศรีจอมทอง นางสาวศิรประภา ทองกอบเพชร นางสาวทิวารัตน์ สินธุวิวัฒน์ นิสิตปริญญาโท นางสาวรัชนีกร บุณยัษฐิติ นิสิตปริญญาตรี นางสาวสุภาพ อรุณภาคมงคล นางสาวอภิรดี เทียมบุญเลิศ นายวีรชัย แก้วผลึก เจ้าหน้าที่วิทยาศาสตร์ โรงพยาบาลจุฬาลงกรณ์ ที่ร่วมทำการทดลองทางห้องปฏิบัติการ

ขอขอบพระคุณสำนักงานส่งเสริมการวิจัย (สกว.) สำนักงานพัฒนาวิทยาศาสตร์และเทคโนโลยีแห่งชาติ (สวทช.) และสำนักงานคณะกรรมการวิจัยแห่งชาติ ที่ให้ทุนสนับสนุน คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย และโรงพยาบาลจุฬาลงกรณ์ ที่สนับสนุนในด้านเงินทุน สถานที่ เจ้าหน้าที่และอื่น ๆ ศาสตราจารย์นายแพทย์ภิรมย์ กมลรัตนกุล คณบดีคณะแพทยศาสตร์ รองศาสตราจารย์แพทย์หญิงรัชนี เซ็นศิริวัฒนา อดีตหัวหน้าภาควิชากุมารเวชศาสตร์ และ

ศาสตราจารย์แพทย์หญิงพิมลรัตน์ ไทยธรรมยานนท์ หัวหน้าภาควิชากุมารเวชศาสตร์ คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ที่ให้การสนับสนุน

และขอขอบคุณผู้ป่วยและครอบครัวที่ให้ความร่วมมือเป็นอย่างดี

บทคัดย่อ

รหัสโครงการ RSA/05/2544

ชื่อโครงการ ความหลากหลายของลักษณะทางคลินิกที่เกิดจากการกลายพันธุ์ของยีน FGFR: จากการเดินโดผิดรูปร่างถึงการเกิดมะเร็ง

ชื่อนักวิจัย รองศาสตราจารย์นายแพทย์วรศักดิ์ โชติเลอศักดิ์

สถาบัน หน่วยเวชพันธุศาสตร์และเมแทบอลิซึม ภาควิชากุมารเวชศาสตร์

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ระยะเวลาโครงการ 1 ธันวาคม 2543 ถึง 30 พฤศจิกายน 2546

โรคในกลุ่มอาการที่มีความผิดปกติทางรูปร่างและกลุ่มโรคพันธุกรรมเมแทบอลิก แม้แต่ละโรคจะพบได้น้อย แต่โดยรวมแล้วนับเป็นสัดส่วนที่สำคัญของการเจ็บป่วย โดยเฉพาะอย่างยิ่งในเด็ก โรคกลุ่มนี้แสดงอาการได้ในทุกอวัยวะ ทุกรูปแบบ ทำให้การวินิจฉัยทำได้ค่อนข้างยาก อย่างไรก็ตามแพทย์จำเป็นต้องพยายามให้การวินิจฉัยที่ถูกต้อง ทั้งนี้เพื่อประโยชน์ในการรักษาผู้ป่วยและป้องกันการเกิดซ้ำของสมาชิกคนอื่นในครอบครัว

คณะผู้วิจัยได้ศึกษาลักษณะทางคลินิก พัฒนาการตรวจทางห้องปฏิบัติการด้วยวิธีการทางชีวเคมีและอณูพันธุศาสตร์เพื่อใช้ในการวินิจฉัยผู้ป่วยไทยที่มีความพิการแต่กำเนิดและโรคพันธุกรรมเมแทบอลิก พบว่าผู้ป่วยไทยหลายโรคมีลักษณะทางคลินิกและการกลายพันธุ์ที่มีลักษณะเฉพาะ รวมทั้ง โรครอยต่อของกะโหลกปิดก่อนกำหนด, กลุ่มอาการ Van der Woude, Pseudoachondroplasia, กลุ่มอาการ Kabuki syndrome, hydrolethalus, และ methylmalonic academia นอกจากนี้ยังพบว่าการกลายพันธุ์บางชนิดนอกจากจะทำให้เกิดความพิการแต่กำเนิดแล้ว ยังเกี่ยวข้องกับกระบวนการเกิดมะเร็งปากมดลูกและมะเร็งหลังโพรงจมูกอีกด้วย

คำหลัก: ความพิการแต่กำเนิด โรคพันธุกรรมเมแทบอลิก มะเร็งปากมดลูก มะเร็งหลังโพรงจมูก การกลายพันธุ์

Abstract

Project Code : RSA/05/2544

Project Title : Clinical variability of *FGFR* mutations: from malformations to malignancies.

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While dysmorphic syndromes and inherited metabolic disorders are individually rare, they collectively account for a significant proportion of illness, especially in children. They present clinically in a wide variety of ways, involving virtually any organ or tissue of the body making them relatively difficult to diagnose. However, reaching an accurate diagnosis for children with dysmorphic features and suspected inherited metabolic disorders is important to them and their families both for treatment and for the prevention of disease in other family members. It also makes available all the accumulated knowledge about the relevant condition.

We studied clinical features, developed biochemical and molecular techniques to help making definite diagnoses for Thai patients with genetic disorders, including dysmorphic syndromes and inherited metabolic disorders. We found out that Thai patients with many of these disorders such as syndromic craniosynostoses, Van der Woude syndrome, Pseudoachondroplasia, Kabuki syndrome, hydrolethalus, and methylmalonic academia, have unique clinical and molecular features. In addition, some of these mutations, besides being responsible for malformation syndromes are related to cervical and nasopharyngeal cancer developments.

Keywords : malformation syndromes, Inherited metabolic disorders, cervical cancer, nasopharyngeal cancer, mutation analysis

เนื้อหางานวิจัย

บทนำ โครงการศึกษาจีโนมมนุษย์ (Human Genome Project) ซึ่งเป็นโครงการทางชีววิทยาที่ใหญ่ที่สุดโครงการหนึ่งในประวัติศาสตร์ของมนุษยชาติ โดยมีจุดประสงค์หลักเพื่อศึกษาการเรียงลำดับ nucleotide ของจีโนมมนุษย์ ได้เสร็จสิ้นลงแล้วเมื่อไม่นานมานี้ โรคของมนุษย์เกือบทุกโรคมีสาเหตุมาจากความผิดปกติของสารพันธุกรรมร่วมกับปัจจัยทางสิ่งแวดล้อม เป็นที่ทราบกันอย่างแน่ชัดว่าลักษณะทางคลินิกของโรคที่มีการถ่ายทอดแบบยีนเดี่ยว เช่น thalassemia ขึ้นกับปัจจัยทางพันธุกรรมเป็นอย่างมาก แต่โรคอื่น ๆ เช่น เบาหวาน, ความดันโลหิตสูง, โรคอ้วน, มะเร็ง รวมทั้งลักษณะนิสัยก็อาจมีส่วนได้รับอิทธิพลจากปัจจัยทางพันธุกรรมด้วย การทราบลำดับ nucleotide ในจีโนมมนุษย์จะทำให้การค้นพบยีนทั้งที่ก่อโรค (disease causing gene) และที่เพิ่มแนวโน้มการเกิดโรค (susceptibility gene) เร็วขึ้นมาก คณะผู้วิจัยจึงได้ถือโอกาสนี้นำความรู้ที่ได้จากโครงการศึกษาจีโนมมนุษย์มาใช้ประโยชน์ในประชากรไทยที่เป็นโรคในกลุ่มความพิการแต่กำเนิด โรคมะเร็งปากมดลูก และโรคพันธุกรรมเมแทบอลิก

ความพิการแต่กำเนิด (congenital anomalies) หมายถึง ความผิดปกติทางรูปร่างแต่กำเนิด เป็นกลุ่มโรคที่มีอุบัติการณ์สูง ทารกแรกเกิด 100 คนจะมีผู้ที่มีความพิการแต่กำเนิดชนิดรุนแรงอยู่ 2 ถึง 4 คน โดยมีโรคและกลุ่มอาการที่เป็นสาเหตุของความพิการเหล่านี้เป็นจำนวนมาก ใน website ที่ชื่อ Online Mendelian Inheritance in Man (OMIM) <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM> ซึ่งบันทึกเฉพาะกลุ่มอาการที่เกิดจากยีนเดี่ยวได้บรรจุรายการไว้มากกว่า 10,000 รายการ เมื่อเกิดความพิการแต่กำเนิดขึ้นแล้ว มักเป็นภาระต่อผู้ป่วย ครอบครัวและสังคมเป็นอย่างมาก การให้การวินิจฉัยที่แน่ชัดได้จากลักษณะทางคลินิกและการกลายพันธุ์จะทำให้แพทย์สามารถให้ข้อมูลแก่ผู้ป่วยและครอบครัวได้อย่างถูกต้อง เช่นการพยากรณ์โรคและโอกาสการเกิดซ้ำ ซึ่งเป็นข้อมูลที่มีความสำคัญมากกับครอบครัวในการตัดสินใจต่อไป นอกจากนั้นความพิการแต่กำเนิดบางชนิดยังสามารถป้องกันเพื่อลดโอกาสการเกิดให้น้อยลงได้ ยิ่งไปกว่านั้น ยีนที่เป็นสาเหตุของความพิการแต่กำเนิดบางยีนยังเกี่ยวข้องกับการเกิดโรคมะเร็งด้วย เช่น *FGFR3* เป็นสาเหตุของการเกิดโรค thanatophoric dwarfism และมะเร็งบางชนิด

โรคพันธุกรรมเมแทบอลิก (inherited metabolic disorders หรือ inborn errors of metabolism) มีความหมายที่ใช้กันโดยทั่วไปคือกลุ่มโรคที่เกิดจากความผิดปกติของกระบวนการย่อยสลาย (catabolism) หรือกระบวนการสังเคราะห์ (anabolism) สารอาหารซึ่งเกิดจากการทำงานของเอ็นไซม์ผิดปกติ การทำงานที่ผิดปกติของเอ็นไซม์จะทำให้เกิดการคั่งของสารตั้งต้นและการขาดของผลิตภัณฑ์ สารตั้งต้นที่คั่งอาจถูกเปลี่ยนไปเป็น metabolites อื่นโดยกระบวนการรอง (minor pathway) ส่ง

ผลให้การทำงานของเซลล์ผิดปกติและเกิดอาการทางคลินิกขึ้น เมื่อเกิดโรคในกลุ่มนี้ ก็เป็นดังเช่น การเกิดความพิการแต่กำเนิด กล่าวคือเป็นโรคที่เป็นภาระต่อผู้ป่วย ครอบครัวและสังคมเป็นอย่างมาก การรักษาให้หายขาดเป็นไปได้ยาก การให้การวินิจฉัยที่แน่ชัดได้จากลักษณะทางคลินิก การตรวจทางชีวเคมีและการกลายพันธุ์จะทำให้แพทย์สามารถให้ข้อมูลแก่ผู้ป่วยและครอบครัวได้อย่างถูกต้อง ซึ่งเป็นข้อมูลที่มีความสำคัญมากกับครอบครัวในการตัดสินใจต่อไป

วัตถุประสงค์

1. เพื่อศึกษาลักษณะทางคลินิกและลักษณะการกลายพันธุ์ของผู้ป่วยไทยที่มีความพิการแต่กำเนิด เช่น craniosynostoses และโรคพันธุกรรมเมแทบอลิก
2. เพื่อศึกษาบทบาทของยีน *FGFR3* ในการเกิดโรคมะเร็งปากมดลูก

วิธีทดลอง

1. ศึกษาลักษณะทางคลินิกของผู้ป่วยไทยที่มีความพิการแต่กำเนิดและเป็นมะเร็งปากมดลูกที่มาที่โรงพยาบาลจุฬาลงกรณ์
2. เก็บเลือด สกัด DNA และศึกษาการกลายพันธุ์ของผู้ป่วย

ผลการทดลอง

คณะผู้วิจัยได้ศึกษาลักษณะทางคลินิกและเก็บตัวอย่างเลือดจากผู้ป่วยและครอบครัว สรุปได้ดังนี้

1. ลักษณะทางคลินิกของผู้ป่วยไทยที่มีความพิการแต่กำเนิดหลายโรคมีลักษณะเฉพาะ
   1.1 Craniosynostosis with skin, eye and joint manifestations[1]
   1.2 Hydrolethalus syndrome อายุยืนกว่าปกติ[2]
   1.3 Kabuki syndrome และมี discordant monozygotic twins คู่แรกของโลก[3]
   1.4 คณะผู้วิจัยศึกษาผู้ป่วยที่เป็นพี่น้อง มีลักษณะอาการทางคลินิกที่เฉพาะ และได้เสนอว่าเป็นกลุ่มอาการใหม่ที่ไม่มีใครรายงานมาก่อน[4]
   1.5 คณะผู้วิจัยได้ใช้วิธีการทางพยาธิวิทยาให้การวินิจฉัยผู้ป่วยโรค Pompe (Glycogen storage disease type II) เป็นจำนวน 2 ราย ซึ่งสามารถนำมาใช้ในการวินิจฉัยก่อนคลอดได้สำเร็จเป็นครั้งแรกในประเทศไทย[5]
2. พัฒนาวิธีการตรวจโรคในกลุ่มที่มีความพิการแต่กำเนิดและโรคพันธุกรรมเมแทบอลิก
   2.1 พัฒนาวิธีการตรวจกรดอินทรีย์ในปัสสาวะด้วยวิธี GC-MS[6]

2.2 คณะผู้วิจัยศึกษาผู้ป่วยที่เป็น methemoglobinemia และยืนยันด้วยวิธีการทางชีวเคมีที่ปฏิบัติในห้องปฏิบัติการในประเทศไทย[7]

2.3 ศึกษาการวินิจฉัย precocious puberty ด้วยการกระตุ้นฮอร์โมน[8]

2.4 พัฒนาการตรวจโรค Achondroplasia ด้วยวิธีทางอณูพันธุศาสตร์[9]

2.5 พัฒนาการตรวจโรค Pfeiffer syndrome ด้วยวิธีทางอณูพันธุศาสตร์[10]

2.6 พัฒนาการตรวจโรค MEN 2A ด้วยวิธีทางอณูพันธุศาสตร์[11]

2.7 ศึกษาลักษณะรูปแบบของกรดอะมิโนในผู้ป่วย cystinuria[12]

2.8 ทบทวนลักษณะทางคลินิก ชีวเคมีและการกลายพันธุ์ในผู้ป่วยไทยที่มีความพิการแต่กำเนิด และโรคพันธุกรรมเมแทบอลิก[13]

3. ลักษณะการกลายพันธุ์ของผู้ป่วยไทยที่เป็นโรคพันธุกรรมหลายโรคต่างจากผู้ป่วยเชื้อสายอื่น เช่น

3.1 Pseudoachondroplasia พบครอบครัวที่มีการกลายพันธุ์ที่เป็นการกลายพันธุ์ใหม่ไม่เหมือนผู้ป่วยอื่นในโลก[14]

3.2 Methymalonic academia พบครอบครัวที่มีการกลายพันธุ์ที่เป็นการกลายพันธุ์ใหม่ไม่เหมือนผู้ป่วยอื่นในโลก[15]

3.3 Van der Woude syndrome พบครอบครัวที่มีการกลายพันธุ์ที่เป็นการกลายพันธุ์ใหม่ไม่เหมือนผู้ป่วยอื่นในโลก[16]

3.4 Crouzon and Apert syndromes พบการกลายพันธุ์ในยีน *FGFR* ซึ่งสามารถนำมาใช้ในการตรวจวินิจฉัยก่อนคลอดได้เป็นผลสำเร็จ[17]

3.5 ผู้ป่วยโรคมะเร็ง คณะผู้วิจัยศึกษาผู้ป่วยหญิงไทยที่เป็น cervical cancer 75 ราย ไม่พบว่ามีการกลายพันธุ์ชนิด S249C ในยีน *FGFR3* ซึ่งต่างจากผู้ป่วยที่เคยมีรายงานมาก่อนในต่างประเทศ ที่บางรายงานมีอัตราการกลายพันธุ์สูงถึงร้อยละ 25 ส่วนการศึกษาผู้ป่วยไทยที่เป็น nasopharyngeal cancer 69 ราย พบว่ามีการกลายพันธุ์ชนิด S249C ในยีน *FGFR3* 1 ราย ซึ่งนับเป็นผู้ป่วย nasopharyngeal cancer รายแรกที่พบการกลายพันธุ์ในยีนนี้[18]

4. คณะผู้วิจัยศึกษาพบว่าลักษณะทางพันธุกรรมแบบหนึ่งในมารดา คือ *MTHFR* 677CT/1298AC เป็นปัจจัยเสี่ยงที่ทำให้บุตรในครรภ์เป็นโรคปากแหว่งเพดานโหว่มากกว่าลักษณะทางพันธุกรรมแบบอื่น ๆ 4.4 เท่า[19]

สรุป

1. โรคความพิการแต่กำเนิดโรคเดียวกันอาจเกิดจากการกลายพันธุ์ต่างชนิดในยีนเดียวกัน หรือจากการกลายพันธุ์ต่างยีนกัน ในขณะที่การกลายพันธุ์ที่เหมือนกันอาจทำให้เกิดโรคที่มีลักษณะทางคลินิกที่แตกต่างกันได้มาก ตั้งแต่ทำให้เกิดความพิการแต่กำเนิดจนถึงการเกิดมะเร็ง

2. ในคนไทยมีกลุ่มอาการบางอย่างที่ไม่เคยมีรายงานมาก่อนในโลก รวมทั้งมีลักษณะทางคลินิก และการกลายพันธุ์ที่แตกต่างจากชนชาติอื่น ซึ่งส่งผลต่อการเลือกวิธีการตรวจวินิจฉัยทางห้องปฏิบัติการ

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Output

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2. การนำผลงานวิจัยไปใช้ประโยชน์

ได้นำผลงานดังกล่าวไปสอนนิสิต และไปเสนอในการประชุมต่าง ๆ ทั่วประเทศ รวมทั้ง จุฬาลงกรณ์มหาวิทยาลัย โรงพยาบาลศิริราช โรงพยาบาลรามาธิบดี สถาบันสุขภาพแห่งชาติมหาราชินี โรงพยาบาลพระมงกุฎเกล้า มหาวิทยาลัยเชียงใหม่ มหาวิทยาลัยขอนแก่น และมหาวิทยาลัยสงขลา

ผลิตนักวิจัยใหม่ ระดับปริญญาโท 4 ท่าน ได้แก่ นายชูพงศ์ อิทธิวุฒิ นายเฉลิมพล ศรีจอมทอง นางสาวศิรประภา ทองกอบเพชร นางสาวทิวารัตน์ สินธุวิวัฒน์

นอกจากนี้งานวิจัยยังเป็นประโยชน์และได้ใช้แล้วกับครอบครัวผู้ป่วยในกลุ่มที่มีความพิการแต่กำเนิดและกลุ่มโรคพันธุกรรมเมแทบอลิกหลายครอบครัว

ภาคผนวก

บทความที่ 1

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Distinct Craniofacial-Skeletal-Dermatological Dysplasia in a Patient With W290C Mutation in *FGFR2*

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Mutations in the fibroblast growth factor receptor genes (*FGFR*) have been known to be associated with many craniosynostosis syndromes with overlapping phenotypes. We studied a 15-year-old Thai boy with an unspecified craniosynostosis syndrome characterized by multiple suture craniosynostoses, a persistent anterior fontanel, corneal scleralization, choanal stenosis, atresia of the auditory meatus, broad thumbs and great toes, severe scoliosis, acanthosis nigricans, hydrocephalus, and mental retardation. Radiography revealed bony ankyloses of vertebral bodies of T9–12, humero-radio-ulnar joints, intercarpal joints, distal interphalangeal joints of fifth fingers, fibulo-tibial joints, intertarsal joints, and distal interphalangeal joints of the first toes. The patient was a heterozygous for a 870G→T change resulting in a W290C amino acid substitution in the extracellular domain of the fibroblast growth factor receptor 2 gene (*FGFR2*). This mutation has previously been reported in a patient with severe Pfeiffer syndrome type 2 that is distinct from the craniosynostosis in our patient. These findings emphasize locus, allelic, and phenotypic heterogeneity of craniofacial-skeletal-dermatological syndrome due to *FGFR2* mutations.

KEY WORDS: fibroblast growth factor receptor 2 gene (*FGFR2*); craniosynostosis; acanthosis nigricans; ankyloses; hydrocephalus; corneal scleralization

INTRODUCTION

At least 100 syndromes are associated with craniosynostosis and are classified based upon clinical features [Muenke and Wilkie, 2001]. Recently, many of the craniosynostosis syndromes, including Crouzon syndrome (MIM 123500), Apert syndrome (MIM101200), Pfeiffer syndrome (MIM 101600), Muenke syndrome (MIM 602849), Jackson-Weiss syndrome (MIM 123150), and Beare-Stevenson syndrome (MIM 123790) were discovered to be associated with mutations in the fibroblast growth factor receptor genes (*FGFR*) [Passos-Bueno et al., 1999]. Here we present a case of unspecified craniosynostosis with an *FGFR2* mutation.

MATERIALS AND METHODS

Clinical Report

The patient was a 15-year-old Thai boy. He was born at full-term by natural spontaneous vaginal delivery after an uncomplicated pregnancy with a birth weight of 2,850 g to a 28-year-old, gravida 2, para 1 mother and her 28-year-old unrelated husband. Family history was unremarkable. At birth, the patient was noted to have craniosynostosis of several cranial sutures, severe midface hypoplasia and noisy breathing. Bilateral inguinal hernias were also noted, which was surgically corrected at age 10 months. At age seven months, a ventriculoperitoneal (VP) shunt was placed for hydrocephalus. At four years, he underwent total calvarial vault reconstruction with fronto-orbital advancement. At approximately 10 years of age, he developed

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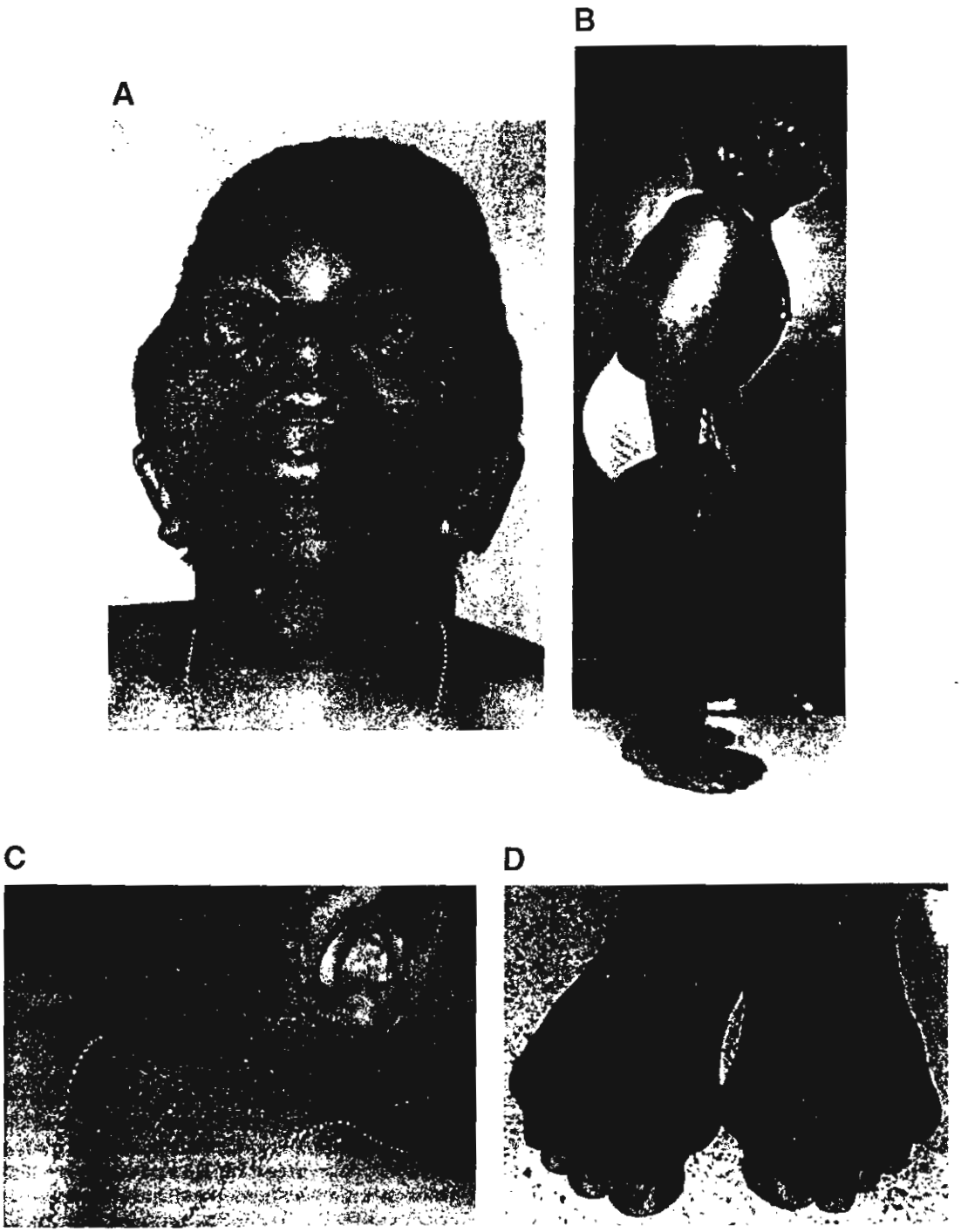
erpigmentation at skin areas and hyperkeratosis sistent with the diagnosis of acanthosis nigricans. Then evaluated by us at age 15 years, physical exaation revealed a weight of 38 kg (-1.5 SD), height 24.5 cm (-6 SD), and OFC of 52 cm (-2 SD). The erior fontanel was still open and measured 0.5 × cm. He had turribrachycephaly, several surgical scars on his scalp, high forehead, depression over the supraorbital ridges and temporal areas, down-slanting palpebral fissures, shallow orbits with severe ocular proptosis, exotropia, bilateral corneal scleralization, and corneal scars with vascularization of corneae and conjunctivae (Fig. 1A–B). Bilateral choanal stenosis, maxillary hypoplasia, severe underbite, inverted-V-



. Postoperative appearance of the patient at age 15 y ars. **A:** Note cloverleaf skull, severe ocular proptosis, scleralization of corneae, low-set ears, rognathia, acanthosis nigricans of periorbital, perinasal, and periorbital areas. **B:** Note abnormal position of joints. **C:** Acanthosis nigricans of the : Broad and laterally deviated halluces.

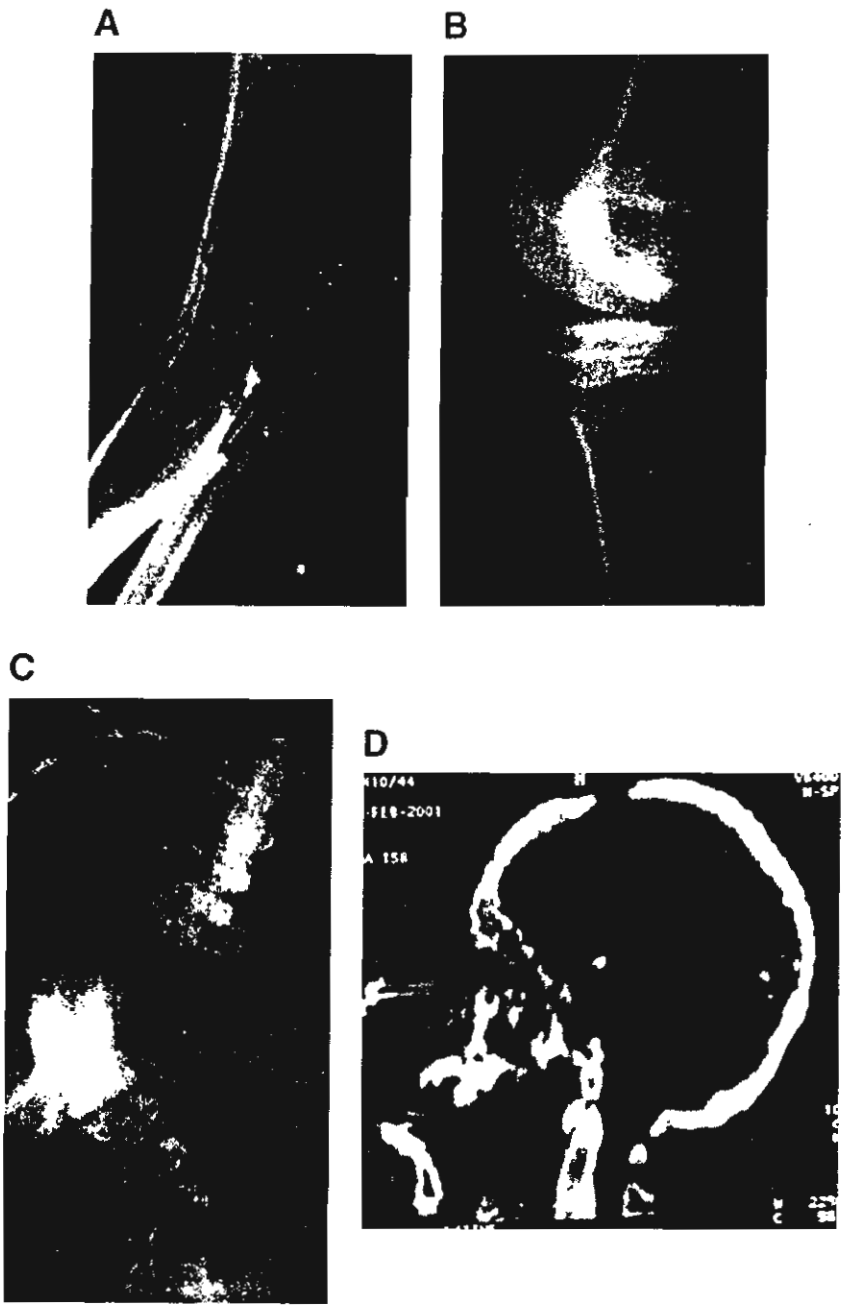


Fig. 2. Radiographs. A: Note complete fusion of the humerus, radius, and ulna prohibiting any motion at the right elbow. B: Tibulofibular fusion at the right knee. C: Severe scoliosis and fusion of vertebral bodies of T9 to T12. D: Brain computer tomography with two-dimension multiplanar reconstruction showing microcephaly and severe maxillary hypoplasia.

shaped palate, and noisy breathing were noted. Ears were low-set and external auditory canals were atretic. Acanthosis nigricans involved the periorbital, perinasal, perioral areas, and neck and axillae (Fig. 1C). The chest wall was asymmetric and severe scoliosis was detected. Examinations of his heart and lungs were unremarkable. His elbows, hips, and knees were fixed at approximately 150 degrees. His thumbs and great toes were broad with the great toe/second toe ratio of 2.0 on the left and 2.12 on the right. The halluces were in valgus position (Fig. 1D). He was unable to talk but able to follow simple commands. His vision and hearing were severely impaired. He had light perception. Brain stem auditory-evoked potentials revealed hearing at approximately 45 and 60 decibels on the right and left, respectively. Electrocardiogram showed no evidence of right heart hypertrophy. Routine laboratory tests were all within the normal limits. Radiography revealed

bony ankyloses of T9–12 vertebral bodies, the humero-radio-ulnar, intercarpal, and distal interphalangeal joints of the fifth fingers and first toes, and of the fibulo-tibial and intertarsal joints (Fig. 2A–C). Computed tomography of the skull and brain revealed microcephaly, hydrocephalus with a ventriculoperitoneal shunt, and severe maxillary hypoplasia (Fig. 2D).

Mutation Analysis

After informed consent was obtained in accordance with the standards set by the local institutional review boards, DNA was extracted from the patient and his parents by a standard method. *FGFR2* exon 8 and *FGFR2* exon 10 were PCR-amplified. Primers, annealing temperatures and PCR procedures were as described previously [Shotelersuk et al., 2001]. PCR products were electrophoresed on a 2% agarose gel (Promega, Madison, WI) and stained with ethidium bromide. DNA on visualized bands was extracted with a kit (Bio 101, Carlsbad, CA), and sequenced in both directions with an automated DNA sequencer (ABI Prism 310 Genetic Analyzer, Perkin Elmer, Foster City, CA).

A heterozygous G → T transversion at nucleotide 870 was identified in *FGFR2* exon 8 of the patient (data not shown). Nucleotide sequence of *FGFR2* exon 10 of the patient, and those of *FGFR2* exons 8 and 10 of his parents, were normal (data not shown).

DISCUSSION

The salient features in our patient were multiple suture craniosynostoses, corneal scleralization, choanal stenosis, atresia of auditory meatus, broad thumbs and great toes, multiple bony ankyloses, severe scoliosis, acanthosis nigricans, hydrocephalus and mental retardation. These clinical features do not fit any known craniosynostosis syndromes (Table I).

Although acanthosis nigricans can be associated with type II diabetes mellitus (DM), patients with acanthosis nigricans and congenital disorders such as Crouzon syndrome did not have DM [Wilkes et al., 1996]. Our patient had a normal fasting glucose level and an oral glucose tolerance test showing no evidence of abnormal glucose metabolism. Craniosynostoses with acanthosis nigricans can result from either of mutations in *FGFR2* (Beare-Stevenson syndrome), or in *FGFR3* (Crouzon syndrome and acanthosis nigricans, CAN) [Passos-Bueno et al., 1999].

Our patient was heterozygous for an 870 G-to-T mutation in *FGFR2*, leading to a substitution of a cysteine for the normal tryptophan at codon 290 (W290C). The mutation was not found in either of his parents, the finding indicating that the mutation was de novo type. The same mutation has previously been reported in a female patient with Pfeiffer syndrome [Schaefer et al., 1998]. Unlike our patient, she had normally formed corneae, a urogenital septum defect, bilateral temporal encephaloceles, patent ductus arteriosus, atrial septal defect, and no fusion of the vertebral, fibulo-tibial,

TABLE I. Clinical Features in Patients With Craniosynostosis

Clinical findings	Our patient	Previously reported patient with W290C	Crouzon with acanthosis nigricans	Pfeiffer syndrome	Crouzon syndrome	Antley-Bixler syndrome	Apert syndrome	Beare-Stevenson syndrome
Craniosynostosis	+	+	+	+	+	+	+	+
Hydrocephalus	+	+	+	+/–	+/–	+/–	+/–	+/–
Acanthosis nigricans	+	–	+	–	–	–	–	+
Broad thumbs and great toes	+	+	–	+	–	–	+	–
Deviation of great toes	Valgus	Varus	–	Varus	–	–	Varus	–
Choanal atresia/stenosis	+	+	+	+/–	–	+	+	+
Atresia of auditory meatus	+	?	+/–	+/–	+	+	–	–
Ankyloses	+	+	–	+/–	–	+	+/–	–
Ocular anterior chamber dysgenesis	+	–	+/–	+/–	+/–	–	+/–	–
Radiographic findings of achondroplasia	–	–	+	–	–	–	–	–
Mental retardation	+	?	+/–	+/–	+/–	+/–	+	?
Early death	–	+	–	+/–	–	+	+/–	+
Inheritance	AD	AD	AD	AD	AD	AR	AD	AD
Mutation	*FGFR2* W290C	*FGFR2* W290C	*FGFR3* A391E	*FGFR1/2* Several	*FGFR2* Several	?	*FGFR2* S252W P253R Others	*FGFR2* S372C Y375C

+, common or present; +/–, occasional; –, uncommon or not present; ?, unknown.

intercarpal, intertarsal, and interphalangeal joints; she died at the age of 10 days. If she had survived to an older age, acanthosis nigricans and bony fusion, which are present in our patient, might have been observed. In contrast to our patient with halluces extended a valgus deviation, the halluces in the patient by Schaefer et al. [1998] were in varus position. Even though clinical manifestations were similar between the two patients with W290C, the natural history was quite different: one died at age 10 days, the other has survived to adolescence. This observation emphasizes phenotypic heterogeneity of the mutation, which could be accounted for by different modifier genes and diverse environmental factors.

Other amino acid changes, e.g., a substitution of tryptophan to arginine or to glycine, at the same codon of *FGFR2* resulted in milder forms of craniosynostosis, such as either classic Crouzon syndrome or an atypical Crouzon syndrome [Oldridge et al., 1995; Park et al., 1995; Meyers et al., 1996; Steinberger et al., 1996]. Cysteine crosslinking forming immunoglobulin-like hoops typifies the region around codon 290 of the fibroblast growth factor receptor [Zhang et al., 1999], and an additional cysteine predicted by the mutation in our patient may lead to aberrant crosslinking and severe changes in the secondary and tertiary structure of the protein.

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บทความที่ 2

Clinical Dysmorphology 2001, 10:51–55

An Asian girl with a 'milder' form of the Hydrolethalus syndrome

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Hydrolethalus syndrome is an autosomal recessive disorder characterized by hydrocephalus, micrognathia, limb anomalies and several other abnormalities, mostly in the midline structures. The syndrome was first described in Finland, where the incidence is approximately 1 in 20 000. All of the Finnish patients were stillborn or died during the first day of life. Only three non-Finnish cases have survived beyond the neonatal period. Here, we report the first Oriental girl with a 'milder' form of hydrolethalus syndrome. The patient died at age 44 days making her the fourth reported case surviving beyond the neonatal period. The case supports the concept of a 'milder' form of the syndrome. Whether this spectrum is due to allelism or locus heterogeneity awaits molecular analysis.
Clin Dysmorphol **10: 51–55**

Keywords: hydrolethalus syndrome

INTRODUCTION

Hydrolethalus syndrome is an autosomal recessive disorder characterized by severe prenatal onset hydrocephalus, a 'key hole-shaped' foramen magnum, hypoplastic eyes, a broad nasal root, cleft palate, malformed low-set ears, micrognathia, limb anomalies, and other abnormalities of the internal organs. It is most common in Finland. Stillbirth or early death is considered to be the rule (Salonen and Herva, 1990). The gene responsible has recently been mapped to chromosome 11q23-25 (Visapaa *et al.*, 1999). Here, we report an Oriental girl with a 'milder' form of hydrolethalus syndrome. The patient had typical craniofacial features except for micrognathia. No internal organ anomalies were found. In addition, the patient survived beyond the neonatal period.

CASE REPORT

A female infant was born at 39 weeks to a 30-year-old G1P0 Thai mother and a 32-year-old unrelated Thai father. There was no known exposure to infections, teratogenic agents, or other environmental hazards. Serology for HbsAg, HIV, and VDRL were negative. The pregnancy was complicated by polyhydramnios. Ultrasonography performed one day before delivery revealed marked hydrocephalus. A cesarean section was performed because of the large head size and breech presentation. Birth weight was 3650 g. Apgar scores were 5 at 1 min and 7 at 5 min. The placenta appeared normal, weighed 480 g, and the umbilical cord length was 34 cm.

The infant had several anomalies including a markedly enlarged head with head circumference of 42 cm,

aplasia cutis of the scalp (1 cm in diameter) on the left side of the parietofrontal area, frontal bossing, malformed low-set ears, hypertelorism, small and deep-set eyes, a poorly formed and bifid nose, a midline cleft upper lip and a cleft palate (Figure 1). The mandible was not small. Her neck was broad in proportion to her shoulders. Examination of the extremities revealed a right clubbed foot. There was no polydactyly or hallux duplex. Cardiac, pulmonary, abdominal and genital examinations were unremarkable.

A diagnosis of hydrolethalus syndrome was made soon after birth. Genetic counselling was provided to the family. Only supportive care including infant formula feeding through an orogastric tube was given to the patient. The patient remained medically stable for 38 days, then started to have respiratory distress, intermittent cyanosis, apnea, and bradycardia and died at age 44 days.

Chromosomal analysis revealed a normal 46, XX karyotype. Ultrasonography of the head, heart, and whole abdomen performed shortly before the patient died revealed only severe hydrocephalus. No cardiac or urinary tract anomalies were identified. Postmortem skull X-rays revealed an abnormal shape of the opening in the base of skull (Figure 2). Radiographs of the other parts of the body were unremarkable. At autopsy the infant weighed 4700 g. Examination of the calvaria showed widely separated sutures and fontanelles. The bony cleft extended posteriorly from the foramen magnum to form an abnormal shape of the opening in the skull base (Figure 2). The brain weighed 570 g and displayed severe hydrocephalus involving the cerebrum but sparing the brain stem and cerebellum. Upon sectioning, the brain showed a dilatation of the third and lateral ventricles but a normal fourth ventricle. Microscopic examination confirmed the presence of aqueduct stenosis. In addition, thinning of corpus callosum, absence of the septum pellucidum and flattening of cerebral cortex were observed. The left eye was severely microphthalmic and the right eye was absent. The other internal organs were anatomically normal.

Table 1 Features of non-Finnish patients with hydrolethalus-like syndrome

Features	Present case	Toriello *et al.* (1985)	Aughton *et al.* (1987)
Polyhydramnios	+	+	+
Preterm delivery	–	+	–
Hydrocephaly	+	+	+
Other CNS malformations	Absence of septum pellucidum	Rudimentary olfactory and optic nerve complex. Diffusely polymicrogyric cerebrum and cerebellum	Porencephaly, encephalocele, hypoplastic cerebellar hemispheres, abnormally shaped medulla, absent septum pellucidum, and a partial defect in the corpus callosum, ventricles open to subarachnoid space
Abnormally shaped foramen magnum	+	+	+
Aplasia cutis	+	–	–
Frontal bossing	+	+	+
Hypertelorism	+	+	N/A
Hypoplastic eyes	+	+	–
Malformed low-set ears	+	+	+
Poorly formed nose	+	+	–
Cleft lip/palate	+	+	Only a small cleft of the secondary palate
Micrognathia	–	+	+
Preaxial polydactyly	–	–	Both feet
Central polydactyly	–	–	–
Post-axial polydactyly	–	–	Both hands
Club feet	+	–	–
Other internal organ involvement	–	Bilateral pulmonary agenesis	Congenital heart defect, genital anomalies
Ethnicity	Thai	Black	European/Lebanon/Amerindian
Consanguineous family history	–	–	–
Age at death	44 days	8 minutes	> 5 months

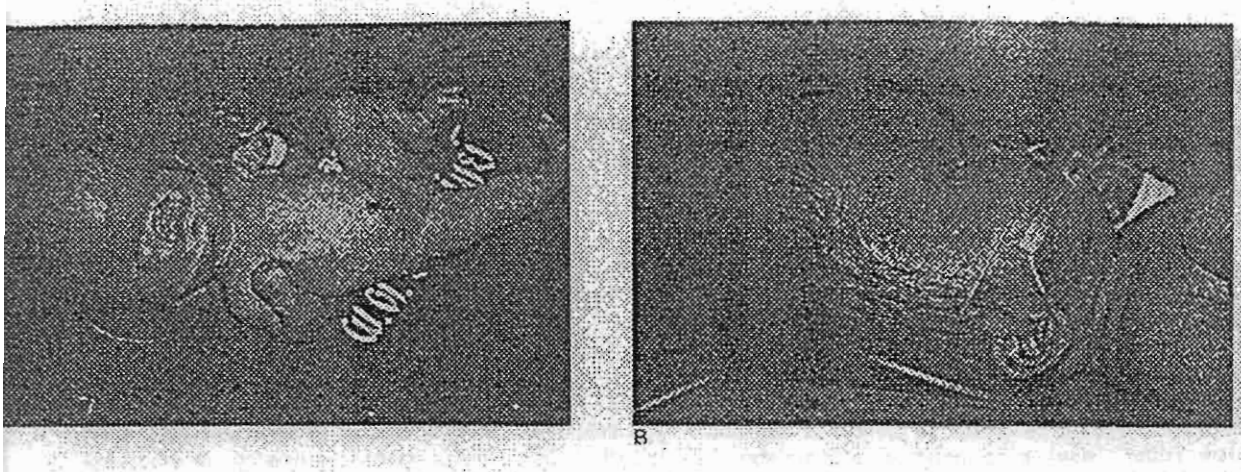
N/A, data not available.

DISCUSSION

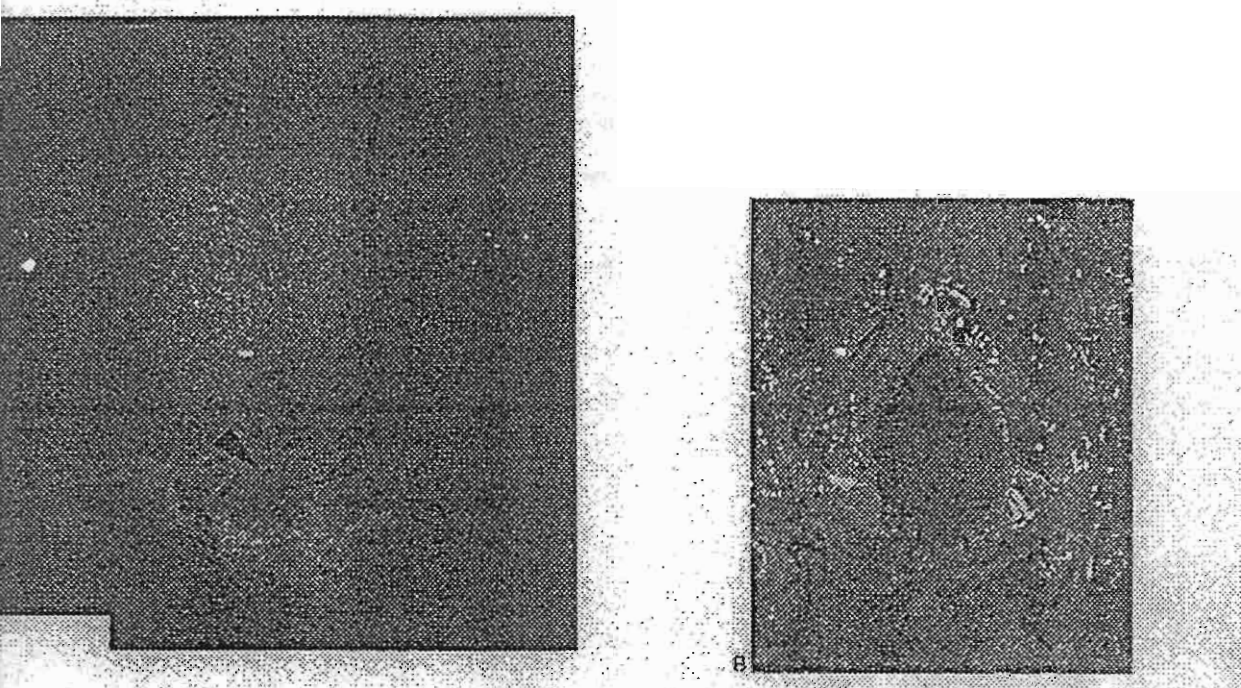
Hydrolethalus syndrome was first described in the early 1980s (Salonen *et al.*, 1981). Since then, more than 90 cases have been reported (Salonen *et al.*, 1981; Adetoro *et al.*, 1984; Toriello *et al.*, 1985; Aughton and Cassidy, 1987; Anyane-Yeboa *et al.*, 1987; Krassikoff *et al.*, 1987; Bachman *et al.*, 1990; Salonen and Herva, 1990; Pryde *et al.*, 1993; Morava *et al.*, 1996; de Ravel *et al.*, 1999; Visapaa *et al.*, 1999). The majority of them (81 cases) are from Finland, where the incidence has been approximated to be at least 1:20 000 (Salonen and Herva, 1990). All of the Finnish cases were stillborn or died during the first day of life (Salonen and Herva, 1990; Visapaa *et al.*, 1999). The syndrome has been reported in other ethnic groups including Afro-Caribbean, Lebanese, Arab, Mexican, and European. Only three cases survived beyond the neonatal period (Table 1).

Hydrolethalus syndrome is currently a clinical diagnosis. Here we report an Oriental female patient who had several major anomalies consistent with the syndrome including severe hydrocephalus from aqueductal stenosis, the 'key hole-shaped' foramen magnum, absence of septum pellucidum, hypoplastic eyes, low-set malformed ears, cleft lip and palate and club foot. However, the patient seemed to have a milder form of the disorder. She did not have micrognathia, previously claimed to be present in 100% of the syndrome. Cardiopulmonary, gastrointestinal, urinary and reproductive systems in this patient were anatomically normal. A previous review article reported congenital heart disease in 46% of patients, defective lobulation of the lungs 62%, abnormal larynx/trachea 58%, urinary tract anomalies 16%, and uterus duplex in females 52% (Salonen and Herva, 1990). In addition, the patient died at age 44 days. There have been only three previously reported cases of hydrolethalus where the infant survived beyond the neonatal period (Aughton and Cassidy, 1987; Anyane-Yeboa *et al.*, 1987; de Ravel *et al.*, 1999). All of them were non-Finnish. Features of non-Finish cases with a milder

Anyane-Yeboa *et al.* (1987)	Morava *et al.* (1996)	de Ravel *et al.* (1999)
N/A	–	+
–	–	–
+	+	+
A Dandy-Walker malformation, absent olfactory bulbs	A Dandy-Walker malformation, agenesis of corpus callosum, absence of cerebellar vermis	A Dandy-Walker malformation, cerebellar hypoplasia, absent corpus callosum
–	+	N/A
–	–	–
+	–	–
+	N/A	N/A
–	–	–
+	Low-set but not malformed	–
+	–	–
+	–	–
+	+	N/A
Both feet	Right hand and both feet	–
Left hand	–	–
–	Right foot and left hand	–
–	–	–
Accessory spleens	–	N/A
Arab	Hungarian	Portuguese/German/South African
+	–	–
17 days (1st case) and 4 months (2nd case)	5 days	7 months



URE 1. The proposita at 1 day of age. (A) Macrocephaly, aplasia cutis, hypertelorism, hypoplastic and deep-set eyes, poorly ned and bifid nose, midline cleft upper lip, broad neck and right clubbed foot. (B) Lateral view of the right side of the face showing -set ear. The mandible was not small



GURE 2. (A). The radiograph of the skull in Towne's view reveals markedly enlarged and thin calvarium. The 'key-hole shaped' amen magnum is outlined in dots and indicated by arrowheads. (B). Gross specimen of the skull base reveals the abnormal shape the foramen magnum. The top of the photograph is the posterior part of the skull

m of hydrolethalus syndrome are presented in Table Hydrolethalus syndrome has been proposed to be herited in an autosomal recessive manner based on a w cases of consanguinity and several families in hich there was recurrence in siblings (Krassikoff, 87). However, history of consanguinity was denied in r family.

The absence of a posterior encephalocele, polydac-ly, and cystic dysplastic kidneys in our patient make the diagnosis of Meckel syndrome unlikely. Acrocallosal syndrome is also unlikely due to the absence of polydactyly and the presence of hydrocephalus in our patient. In addition, our patient's features were not consistent with any other known syndromes. Another possibility is our patient represents a distinct syndrome with major features including aqueductal stenosis, microphthalmia, and median cleft lip.

This case supports the concept of a 'milder' form of the syndrome and the founder effect in the Finnish population for the severe form. Whether this spectrum is due to allelism or locus heterogeneity awaits molecular analysis.

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บทความที่ 3

American Journal of Medical Genetics 110:384–390 (2002)

Clinical Report

Kabuki Syndrome: Report of Six Thai Children and Further Phenotypic and Genetic Delineation

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We describe six Thai children with the Kabuki syndrome. Monozygotic twin boys discordant for the syndrome were encountered in a family. The affected twin had all five cardinal features of the syndrome, whereas the unaffected twin had none of them. The presence of monozygotic twins discordant for the syndrome argues against a single gene origin of the disorder, but by no means excludes it. In another family, a mother had a facial appearance similar to her affected son. Lower lip pits with or without symmetrical lower lip nodules were present in three of the six children, and pilonidal sinus was seen in five children. These clinical manifestations were much more common than previously described. Other inconsistent findings included early eruption of the lower central incisors, a skin defect of the head, and transient hyperthyrotropinemia in infancy.

KEY WORDS: **Kabuki syndrome; lower lip pits; pilonidal sinus; hyperthyrotropinemia; aplasia cutis; discordant twins**

INTRODUCTION

Kabuki syndrome (KS), first described in 1981 [Kuroki et al., 1981; Niikawa et al., 1981], is characterized by peculiar facies, dermatoglyphic abnormalities with persisting fingerpads, skeletal anomalies, mild to moderate mental retardation, and postnatal growth deficiency [Niikawa et al., 1988]. To our knowledge, a total of 246 patients have been reported in the literature. Several possible etiologies have been proposed, including chromosomal abnormalities [Fryns et al., 1994] and an autosomal dominant disorder with variable expressivity [Courtens et al., 2000]. However, the true cause of the syndrome remains unknown.

Here we describe six Thai children with KS. Some of their clinical manifestations are much more common than described previously, and a few have not been previously described. Monozygotic twin boys discordant for the syndrome are described.

SUBJECTS AND METHODS

Six children with KS were identified and studied in the Genetics Clinic of the King Chulalongkorn Memorial Hospital during the 2-year period from July 1999 to June 2001. Parents of all six patients were nonconsanguineous.

Patient 1

Twin boys were born at 32 weeks of gestation to a 20-year-old G1P0 mother and a 33-year-old father. Besides the twinning, the pregnancy and labor were uncomplicated. There was no history of fever, rash, spotting, tobacco, alcohol, illicit drug use, or x-ray exposure during the pregnancy. The twins stayed in the hospital for 7 weeks and 9 weeks, respectively, after birth.

Twin A walked and spoke at age 14 months. At his last visit at age 9 years, he was ranked 22nd of 43 students in his third-grade class and measured 125.8 cm (−0.5 SD), weighed 19.9 kg (−1.5 SD), and had an OFC of 51 cm (−1 SD). Physical examination was unremarkable (Fig. 1).

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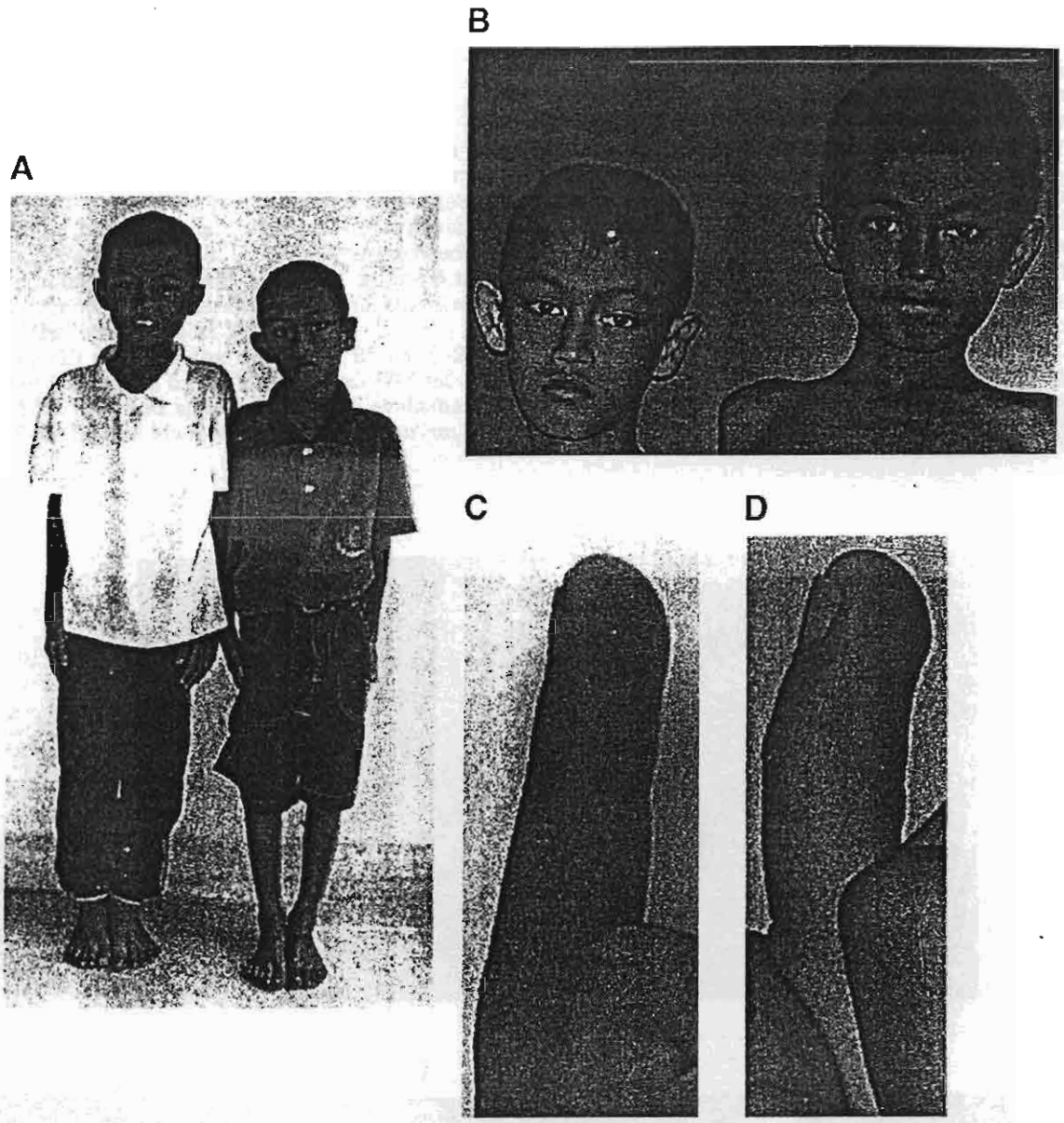

Fig. 1. Patient 1 and his monozygotic twin. A: Growth failure in twin B (right). B: Long palpebral fissures and large and prominent ears in twin B (left). C: Left fourth finger of twin A (normal). D: Finger pad in twin B.

Twin B (patient 1) first rolled over at age 12 months, sat at 2 years, crawled at 3 years, walked at 4 years, and spoke at 6 years. At age 9 years, his vocabulary was approximately 10 words, and he could follow simple commands, run, and walk up and down stairs. Besides a febrile seizure at age 4 years, he had been healthy. The twins' father and two uncles also had histories of febrile seizures. At age 9 years, he measured 116 cm (−2 SD), weighed 16 kg (−2 SD), and had an OFC of 49 cm (−3 SD). He showed growth failure (Fig. 1A), long eyelashes, ectropion of the lateral lower eyelids, and large and prominent ears (Fig. 1B). His right ear was malformed and his left ear had a scar across the pinna. He had a short linear scar on his abdominal wall above his umbilicus. He had brachydactyly and prominent finger pads of bilateral third and fourth fingers (Fig. 1C). His IQ was estimated at 59. He had bilateral severe sensorineural hearing loss. Bone age at the chronological age of 8½ years was 6 years. Chromosomes were normal: 46,XY. *FMR1* methylation PCR analysis for fragile X syndrome was negative. Thyroid function tests (thyroxine [T4] and thyroid-stimulating hormone [TSH]) at age 9 years were normal.

Analysis of microsatellite markers was performed in the twins as previously described [Shotelersuk et al., 1999]. Thirteen markers on 11 chromosomes were inter-

pretable and identical in the twins. The affirmative probability for monozygosity in the twins was estimated at more than 99.9%.

Patient 2

A girl was born at 34 weeks' gestation with a birth weight of 2,675 g to a 34-year-old G3P2 mother and a 29-year-old father. She was hospitalized at ages 6 days and 5 months due to gastroenteritis and again at age 9 months for pneumonia. Lower central incisors erupted at 4 months. She held her head at age 4 months, rolled over at 7 months, sat at 11 months, walked at 16 months, and spoke at 18 months.

At age 10 months, she measured 65 cm (−2.5 SD), weighed 5.2 kg (−2.5 SD), and had an OFC of 40.5 cm (−2.5 SD). She had sparse hair, long eyelashes, eversion of the lateral lower eyelids, hypertelorism, prominent ears, a depressed nasal tip, and micrognathia (Fig. 2A). There were two symmetrical nodules with a pit in the center of each nodule on her lower lips with discharge (Fig. 3A). Her narrow, high-arched palate with incomplete cleft was surgically corrected at 15 months. She had a right single transverse crease, brachydactyly, single creases and clinodactyly of bilateral fifth fingers, and prominent finger pads. A pilonidal sinus was present. Her developmental quotient (DQ) was 78. She had epiphora due to obstruction of bilateral lacrimal ducts. Bone age was normal. The serum TSH level was increased (8.94 μU/mL; normal, 0.3–4.1 μU/mL) at age 10 months but normalized at 18 months, and T4 and free thyroxine (FT4) were normal at both ages. Immunoglobulin A (IgA) was decreased (< 22.5 mg/dL; normal, 30–180 mg/dL), while other immunoglobulins were normal. Her and her parents' chromosomes were normal.

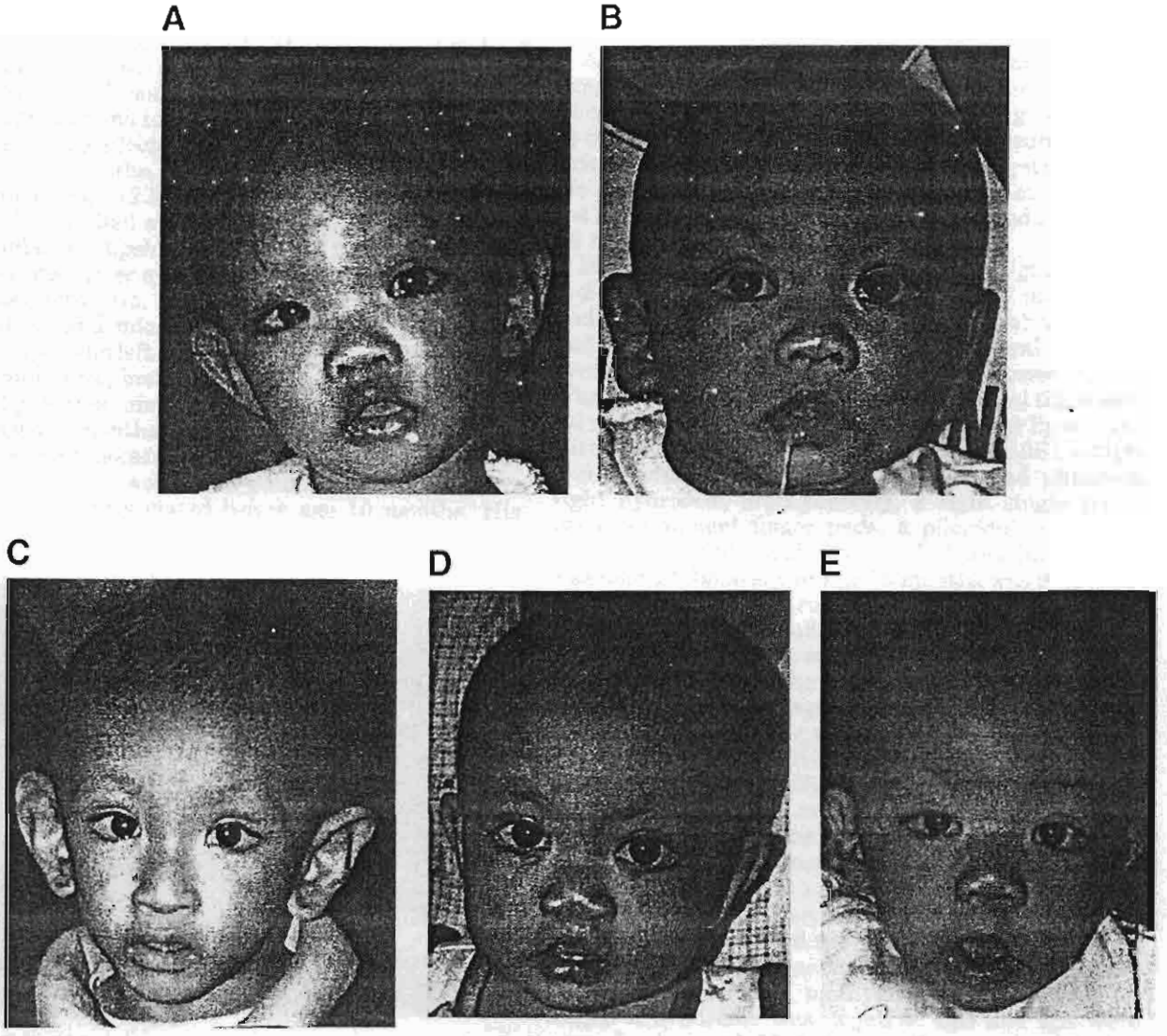

Fig. 2. Facial appearance of patients 2 (A), 3 (B), 4 (C), 5 (D), and 6 (E).

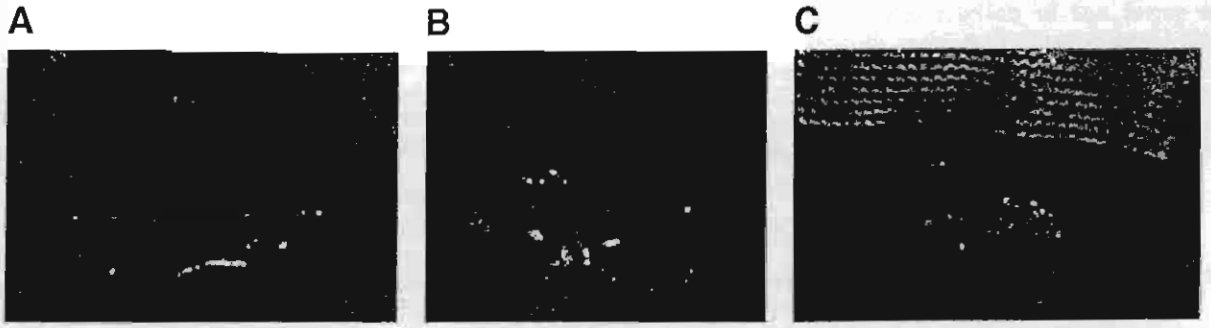

Fig. 3. Symmetrical lower lip nodules with central pits in patients 2 (A) and 4 (B), and lower lip pits without nodules in patient 5 (C).

Patient 3

A boy was born after uncomplicated pregnancy at term with a birth weight of 2,900 g to a 24-year-old G1P0 mother and a 25-year-old father. Lower central incisors erupted at age 2 weeks. At 2 months, he was hospitalized for pneumonia. He held his head at 3 months and rolled over at 4 months. His mother had long palpebral fissures and bowed eyebrows but had normal growth and intelligence (Fig. 4). No other family members were affected.

At age 4 months, he measured 59 cm (−1.5 SD), weighed 4.3 kg (−2.5 SD), and had an OFC of 37.5 cm (−2.5 SD). He had sparse lateral one-third eyebrows, long eyelashes, upslanted palpebral fissures, eversion of the lateral lower eyelids (Fig. 2B), prominent ears, a depressed nasal tip, two lower central incisors, a cleft soft palate, and micrognathia. A grade II/VI systolic murmur over the left chest wall was present. He had an umbilical hernia, brachydactyly, and prominent finger pads. A pilonidal sinus was present. His DQ was 97. Bone age at 4 months was appropriate for his chronological age. An echocardiography revealed a mild form of coarctation of the aorta and patent ductus arteriosus that spontaneously closed before age 10 months. His chromosomes and immunoglobulin levels were normal. FT4 at age 4 months was normal, but TSH was slightly high (4.49 μU/mL; normal, 0.3–4.1 μU/mL).

Fig. 4. Patient 3 and his parents. Note the facial similarity between the patient and his mother.

Patient 4

A boy was born at term after an uncomplicated pregnancy to a 34-year-old G2P1 Thai mother and a 35-year-old father. Birth weight was 3,500 g. After birth to the age 2 8/12 years, he had frequent pneumonia and urinary tract infections requiring 13 hospitalizations. He held his head at 3 months, rolled over at 5 months, sat at 14 months, spoke at 14 months, pulled to stand at 18 months, and walked at 26 months.

His length was 85 cm (−1.5 SD), weight 9,700 g (−2 SD) and OFC 45 cm (−3 SD) at age 32 months. He had sparse hair and lateral one-third eyebrows, long and thick eyelashes, upslanted palpebral fissures, eversion of the lateral portion of the lower eyelids (Fig. 2C), prominent ears, a depressed nasal tip, bilateral preauricular pits, two symmetrical lower lip nodules with a pit in the center of each nodule (Fig. 3B), a high-arched palate, and micrognathia. He had phimosis, right hydrocele, brachydactyly, a right single transverse, prominent finger pads, a pilonidal sinus, and mild generalized hypotonia. His DQ was 66. Hearing was normal. Bone age at age 15 months was 9 months. Renal ultrasound, intravenous pyelography, voiding cystourethrography, and cortical scintigraphy (Tc-99m 2,3-dimercaptosuccinic acid [DMSA]) revealed a double collecting system of the left kidney with moderately dilated pelvicalyceal system and vesicouretororenal reflux of the left lower moiety. The double collecting system and ureteropelvic junction obstruction of the left kidney was surgically corrected at age 2 5/12 years. Echocardiography and cardiac catheterization revealed a moderate-size perimembranous ventricular septal defect (VSD) with pulmonary hypertension. The VSD was surgically closed at age 13 months. Due to recurrent pneumonia, computerized tomography of the chest was performed and revealed normal lung parenchyma and airways. His G-6-PD was deficient. Chromosomes were normal: 46,XY. *FMR1* methylation PCR analysis for fragile X syndrome was negative. IgG and IgA were normal, whereas IgM (169.3 mg/dL; normal, 36.8–144 mg/dL) was slightly increased.

Patient 5

A girl was born at term to a 29-year-old G2P1 mother and a 31-year-old father. The pregnancy was uncomplicated. Birth weight was 2,400 g. She held her head at age 3 months, rolled over at 6 months, but was unable to grasp things or sit at 7 months.

At age 7 months, she measured 63 cm (−1.5 SD), weighed 6.0 kg (−2 SD), and had an OFC of 42.5 cm (mean). She had arched and sparse lateral one-third eyebrows, long and thick eyelashes, eversion of the lateral portion of the lower eyelids (Fig. 2D), prominent ears, a depressed nasal tip, bilateral complete cleft lip (surgically corrected at age 7 months) and palate, and micrognathia. There were lower lip pits without nodules (Fig. 3C). She had brachydactyly, clinodactyly, and hypoplastic middle phalanges of the fifth fingers, prominent finger pads, a pilonidal sinus, and a hairy hyperpigmented macule. A café-au-lait spot (0.5 cm) was present at the left abdominal wall. Her DQ was 71. Chromosomes were normal.

Patient 6

A boy was born at term with a birth weight of 3,030 g to a 29-year-old G2P1 mother and a 46-year-old father. The pregnancy was complicated by maternal gestational diabetes mellitus, which could be controlled by diet alone. He had neonatal hypoglycemia requiring intravenous glucose for a few days. Lower central incisors were erupted at age 4 months. He held his head at age 4 months, but was still unable to roll over at 5 months.

At age 5 months, he measured 65 cm (mean), weighed 7.4 kg (+0.5 SD), and had an OFC of 40 cm (−1 SD). He had aplasia cutis (0.8 cm) on the occipital area, sparse lateral one-third eyebrows, long and thick eyelashes, eversion of the lateral portion of the lower eyelids (Fig. 2E), prominent ears, a depressed nasal tip, bilateral preauricular pits, two lower central incisors, a narrow and high-arched palate, and a cleft uvula. He had brachydactyly, clinodactyly and single creases of the fifth fingers, prominent finger pads, a pilonidal sinus, and a hairy hyperpigmented macule.

DISCUSSION

We described six Thai children with KS and some interesting clinical and genetic characteristics. All six children showed the five cardinal manifestations of the syndrome (Table I), with the exceptions of patient 6, with normal weight and length at age 5 months, and patient 3, with normal development at age 7 months. They included typical peculiar facies, dermatoglyphic abnormalities with persisting fingerpads, skeletal anomalies including brachydactyly, mild to moderate mental retardation, and postnatal growth deficiency. The growth of patient 6 may yet prove deficient in the second half of infancy, like other patients with typical KS [Niikawa et al., 1988]. Other associated abnormalities in the six children included cleft lip/cleft palate (patients 2, 3, and 5) [Burke and Jones, 1995], congenital heart defects (patients 3 and 4), hearing loss (patient 1) [Igawa et al., 2000], delayed bone age (patient 1) [Niikawa et al., 1988], kidney anomalies (patient 4), and abnormal immunoglobulin levels (patient 2) [Hostoffer et al., 1996].

Three (patients 2, 4, and 5) of six patients we described had lower lip pits with occasional clear discharge. Patients 2 and 4 also had lower lip nodules. *The nodules of both patients were symmetrical. Of 246 patients reported with KS, only four had lower lip pits* [Franceschini et al., 1993; Kokitsu-Nakata et al., 1999; Makita et al., 1999]. The lower lip pits are also present

TABLE I. Clinical and Laboratory Findings in Six Children*

	Patient number							
Clinical findings	1	2	3	4	5	6	Total	Niikawa et al. [1988]
Sex	M	F	M	M	F	M		M:F = 1:1
Age (years)	9	1	7/12	2	9/12	5/12		
Growth failure	+	+	+	+	+	−	5/6	73%
Developmental delay	+	+	−	+	+	+	5/6	92%
Typical face	+	+	+	+	+	+	6/6	100%
Lower lip pits and nodules	−	+	−	+	+	−	3/6	
Cleft palate	−	+	+	−	+	−	3/6	41%
Early eruption of lower central incisors	−	+	+	−	−	+	3/6	
Congenital heart disease	−	−	+	+	−	−	2/6	32%
Fingertip pads	+	+	+	+	+	+	6/6	78%
Skeletal abnormalities	+	+	+	+	+	+	6/6	92%
Pilonidal sinus	−	+	+	+	+	+	5/6	
Hearing loss	+	−	−	−	NA	NA	1/4	24%
Urinary tract anomalies	−	−	NA	+	NA	−	1/4	12%
Abnormal Ig levels	NA	+	−	−	NA	NA	1/3	
Normal chromosomes	−	−	−	−	−	NA	5/5	
Hyperthyrotropinemia in infancy	NA	+	+	NA	NA	NA	2/2	
Others						Cleft lip	Aplasia cutis	

*NA, data not available.

n van der Woude syndrome (VWS) (MIM 119300). The ossibility that KS could be caused by a microdeletion, ncluding the VWS type 1 critical region at 1q32-q41, as been excluded [Makita et al., 1999].

Pilonidal dimples have been described with KS Franceschini et al., 1993; Makita et al., 1999], but not n the 62 patients reported by Niikawa et al. [1988]. ecause pilonidal dimples were present in five (patients -6) of six patients, with two of them (patients 5 and 6) ith hairy hyperpigmented macules on the sacral area, e propose that this finding is a part of clinical mani-stations of KS, and probably related to sagittal cleft-g of vertebrae [Niikawa et al., 1988].

The lower central incisors erupted before age months in three of six patients (2 weeks, 4 months, nd 4 months for patients 3, 2, and 6, respectively). lthough dental anomalies are frequent and variable in atients with the syndrome [Matsune et al., 2001], to ur knowledge, early eruption of the two lower central cisors has not been described.

Several hormonal abnormalities have been described KS, including growth hormone (GH) deficiency, evated follicle-stimulating hormone (FSH) and pro-ctin [Niikawa et al., 1988; Franceschini et al., 1993], ntral diabetes insipidus [Tawa et al., 1994], and ngenital hypothyroidism [Kawame et al., 1999]. We served hyperthyrotropinemia in two of the three tients examined for this complication (patients 2 and . The increased TSH was normalized after infancy in tient 2.

The mother of patient 3 had a facial appearance miniscent of KS (Fig. 4). Kuroki et al. [1981] sugges-d that the syndrome is inherited as an autosomal minant trait in which affected individuals represent w mutations. Dominant inheritance of the syndrome as supported by more than 10 families in which ere was a facial resemblance between a patient and e parent [Niikawa et al., 1988; Halal et al., 1989; ina et al., 1995; Kobayashi and Sakuragawa, 1996; engo et al., 1996; Tsukahara et al., 1997; Wilson, 98; Courtens et al., 2000].

Monozygotic twin boys concordant for KS have been orted, and have reinforced the belief that KS has a netic basis [Lynch et al., 1995]. On the other hand, r patient 1 had an unaffected monozygotic twin. tient 1 had all five cardinal features of KS, whereas unaffected twin had none. Their monozygosity was pported by molecular studies. The presence of dis-dant monozygotic twins argues against, but by no ans excludes, monogenic disorders. There have been ny instances of monozygotic twins discordant for mo-genic disorders, including spondylocostal dysostosis n Thienen and van der Auwera, 1994] and oral-ial-digital syndrome type 1 [Shotelersuk et al., 1999]. eral causes for such discordance have been sugges-, including postzygotic, posttwinning, and somatic tation [Shotelersuk et al., 1999].

n summary, we described six Thai children with KS. ver lip pits with or without symmetrical lower lip ules and pilonidal sinus were much more common ur series than previously described. Early eruption he two lower central incisors, transient hyperthyr-otropinemia in infancy, and aplasia cutis were observed. A mother had a facial appearance similar to her affected son. However, we also described discordant monozygotic twin boys with KS, arguing against autosomal dominant inheritance.

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บทความที่ 4

American Journal of Medical Genetics 116A:164–169 (2003)

New Syndrome?

Postnatal Growth Failure, Microcephaly, Mental Retardation, Cataracts, Large Joint Contractures, Osteoporosis, Cortical Dysplasia, and Cerebellar Atrophy

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We describe two sibs with postnatal-onset growth deficiency, microcephaly, cataract, prominent supraorbital ridge, large joint contractures, severe osteoporosis, cortical dysplasia, cerebellar atrophy, and mental retardation. The combination appears to constitute a previously undescribed syndrome inherited in an autosomal recessive pattern. © 2003 Wiley-Liss, Inc.

KEY WORDS: **growth failure; mental retardation; microcephaly; cataract; arthrogryposis; osteoporosis; cortical dysplasia; cerebellar atrophy**

INTRODUCTION

There have been many MCA/MR syndromes with a combination of short stature, microcephaly, cataracts and mental retardation. These microcephalic dwarfism with cataract include CAMAK or CAMFAK syndrome (MIM 212540) (cataract, microcephaly, failure to thrive, arthrogryposis, and kyphoscoliosis) [Talwar and Smith, 1989]; cerebro-oculo-facio-skeletal (COFS) syndrome (MIM 214150) (growth failure, microcephaly, severe mental retardation, microphthalmia, cataracts, prominent nose, large ears, progressive joint contractures, camptodactyly, osteoporosis, and intracranial calcifications) [Pena and Shokeir, 1974]; microcephalic primordial dwarfism, Toriello type (MIM 251190) (growth deficiency, microcephaly, cataracts, mental retardation, enamel hypoplasia, immune deficiency, and delay of ossification) [Toriello et al., 1986]; Warburg micro syndrome (MIM 600118) (microcephaly, microcornea, cataracts, mental retardation, optic nerve atrophy, prominent nose, large ears, hypogenitalism, and hypotonia) [Warburg et al., 1993]; Martsolf syndrome (MIM 212720) (short stature, severe mental retardation, cataracts, hypogonadism, hypotonia, lax joints, and osteoporosis) [Harbord et al., 1989]; and ataxia-microcephaly-cataract (AMC) syndrome (MIM 208870) (ataxia, microcephaly, hypotonia, mental retardation, and cataracts) [Ziv et al., 1992].

We report on two children (a girl and a boy) in a sibship of four with another form of microcephalic dwarfism with cataract that is distinct from previously described syndromes.

CLINICAL REPORTS

Patient 1

The proband, a Thai girl, was the third child of a 31-year-old father and a 30-year-old mother. The parents were healthy and unrelated. Their first two children were normal and no other family members had short stature, mental retardation, or congenital anomalies. The proband was born at term after a normal pregnancy with a birth weight of 3,050 g (50th centile) and birth length of 51 cm (50th centile). Her OFC at birth was noted to be normal but the actual size was not available. No abnormalities were noted at birth. Since infancy, she gained weight poorly. Her weight at ages 2 months, 6 months, and 1.5 years were 3.9 kg (−2 SD), 4.8 kg (−3 SD), and 6.0 kg (−4 SD), respectively. Her

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length at the same ages were 55 cm (mean), 60 cm (−2 SD), and 66 cm (−5 SD), respectively. Her development had been delayed. She first rolled over at 4 1/12 years. At 9 years of age, she crawled, had to be held to stand, and walked without support for only 2–3 steps. She could build towers of seven blocks and follow simple commands. She had a vocabulary of approximately 10 words. Her deciduous and permanent teeth were normally erupted and developed. Her large joints were noted to have limitation of movement beginning at around 2 years of age. Besides bilateral lenticular cataracts, which were detected and extracted at 8 years, her general health had been unremarkable. She had never suffered from seizures or severe infections.

On physical examination at age 9 years, her weight was 13 kg (−4 SD), height 99.5 cm (−6 SD), and OFC 44.5 cm (−5 SD). She had prominent supraorbital ridge, prominent nasal root, and wide mouth (Fig. 1A). Her eyes were not deep-set, and teeth were normal. There were no abnormalities of the chest wall, genitalia, hands, or feet. Dermatoglyphics of her left and right hands from the first digit to the fifth digit were AUUUW and UAAUA, respectively. Her back was without scoliosis. Limitation of motion of the shoulders, elbows, hips and knees were noted: her elbows and knees could be extended to a maximum of approximately 170 degrees (Fig. 2A), but the wrist, ankle, and phalangeal joints were normal. There was no spasticity, and her motor power was normal with DTR of 1+. Plantar reflexes were down-going.

Besides bilateral cataracts, ophthalmologic examination showed normal anterior segment, fundus, retina, and the maculae. Hearing tests by pure tone audiogram (evaluated by head turning to a sound source) and brain stem auditory evoked potentials were normal. Developmental assessment by the Gesell Developmental Schedule showed a mental age of 20 months at a chronological age of 9 3/12 years. Other than severe osteoporosis, a skeletal survey was unremarkable. There was no kyphoscoliosis and the acetabular roofs were well formed without hip dislocation. Brain MRI showed microcephaly predominantly affecting the frontal lobes and marked cerebellar atrophy with possible atrophy of the pons, medulla and upper cervical cord (Fig. 3A). There were also multiple focal areas of abnormally thickened cortex of bilateral frontal and right parietal lobes and multiple small scattered hyperintense foci on T2WI and FLAIR images in subcortical white matter close to the vertex, suggestive of cortical dysplasia and the presence of gliosis in the underlying subcortical white matter. Cavum septum pellucidum was present. Myelination of white matter and corpus callosum appeared normal. She had a normal 46,XX karyotype. Serum levels of IgG (1,847 mg/dl; normal, 600–1,600 mg/dl) and IgM (285.7 mg/dl; normal, 38.4–148 mg/dl) were slightly increased, whereas that of IgA was normal. Serum levels of T4, free T4, TSH, FSH, LH, estradiol are all normal.

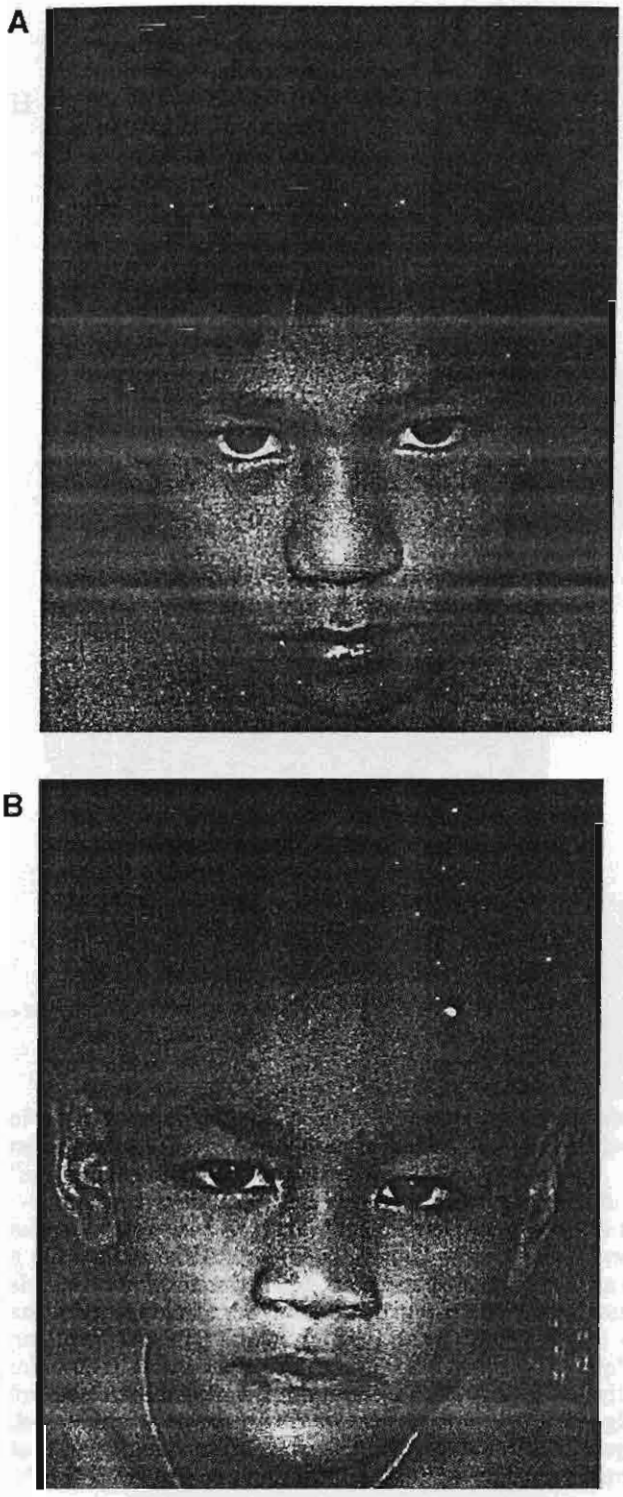

Fig. 1. The elder sister (A) and the younger brother (B). Note the triangular facies with prominent supraorbital ridge and prominent ears.

Patient 2

The younger brother of Patient 1 (P1) was delivered at term after a normal gestation. His birth weight was 2,900 g (−0.5 SD) with an unremarkable physical examination. His weight at ages 2 months, 6 months, and 1.5 years were 4.3 kg (−1 SD), 5.6 kg (−3 SD), and 6.5 kg (−4 SD), respectively. His length and OFC in

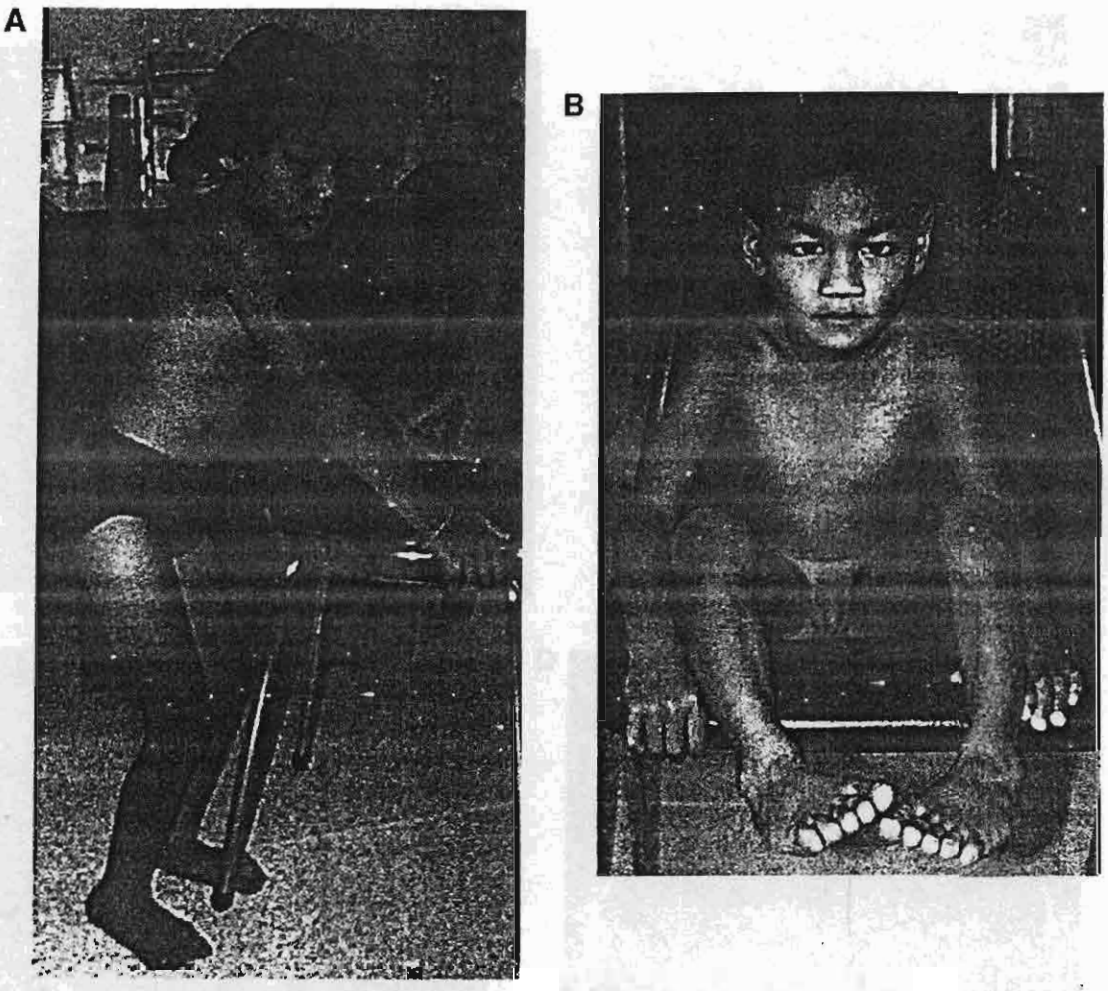

Fig. 2. Joint contractures, especially of the hip, knees and elbows in the sister (A), and normal male genitalia in the brother (B).

infancy were unavailable. His development had been delayed. At 6 years, he began to walk with some support, could follow simple commands but had no speech. Bilateral lenticular cataracts were diagnosed at age 3 years and removed. He developed tonic-clonic seizures at age 5 years. The frequency of the seizures was approximately twice a year. Except for an episode of acute diarrhea in infancy, he had never had a severe infection.

On physical examination at age 6 years, his weight was 10.5 kg (−4 SD), height 90.5 cm (−6 SD), and OFC 44 cm (−5 SD). He had a similar facial appearance to that of P1 but distinct from those of two other unaffected sibs. He had triangular facies, prominent supraorbital ridge, prominent ears, and prognathism (Fig. 1B). His genitalia was of a normal prepubertal male appearance (Fig. 2B). Dermatoglyphics of his left and right hands from digits 1–5 were WWUWW and WUUWW, respectively. The motion of his large joints was more severely limited than that of P1. His elbows and knees were extended to a maximum of approximately 160 and 150 degrees, respectively. Neurological examination showed normal muscle power, no spasticity, and DTR of 1+. Unlike his affected sister, he had positive bilateral ankle clonus and up going plantar reflexes. All other findings were similar to those of P1.

Developmental assessment by the Gesell Developmental Schedule showed a mental age of 13 months at a chronological age of 6 5/12 years. A skeletal survey showed severe osteoporosis without scoliosis. Results of an ophthalmologic examination and hearing tests were normal. Electroencephalography showed evidence of mild diffuse encephalopathy with no definite epileptiform activity. Brain MRI was similar to that of P1 with the addition of a 2 × 3 cm arachnoid cyst in the right temporal area (Fig. 3B–D). Proton MR spectroscopy of the occipital white matter showed normal spectrum.

DISCUSSION

The two sibs we described had a similar combination of malformations, i.e., postnatal growth failure, microcephaly with cortical dysplasia and cerebellar atrophy, severe mental retardation, prominent supraorbital ridge, cataracts, limitation of movement of the large

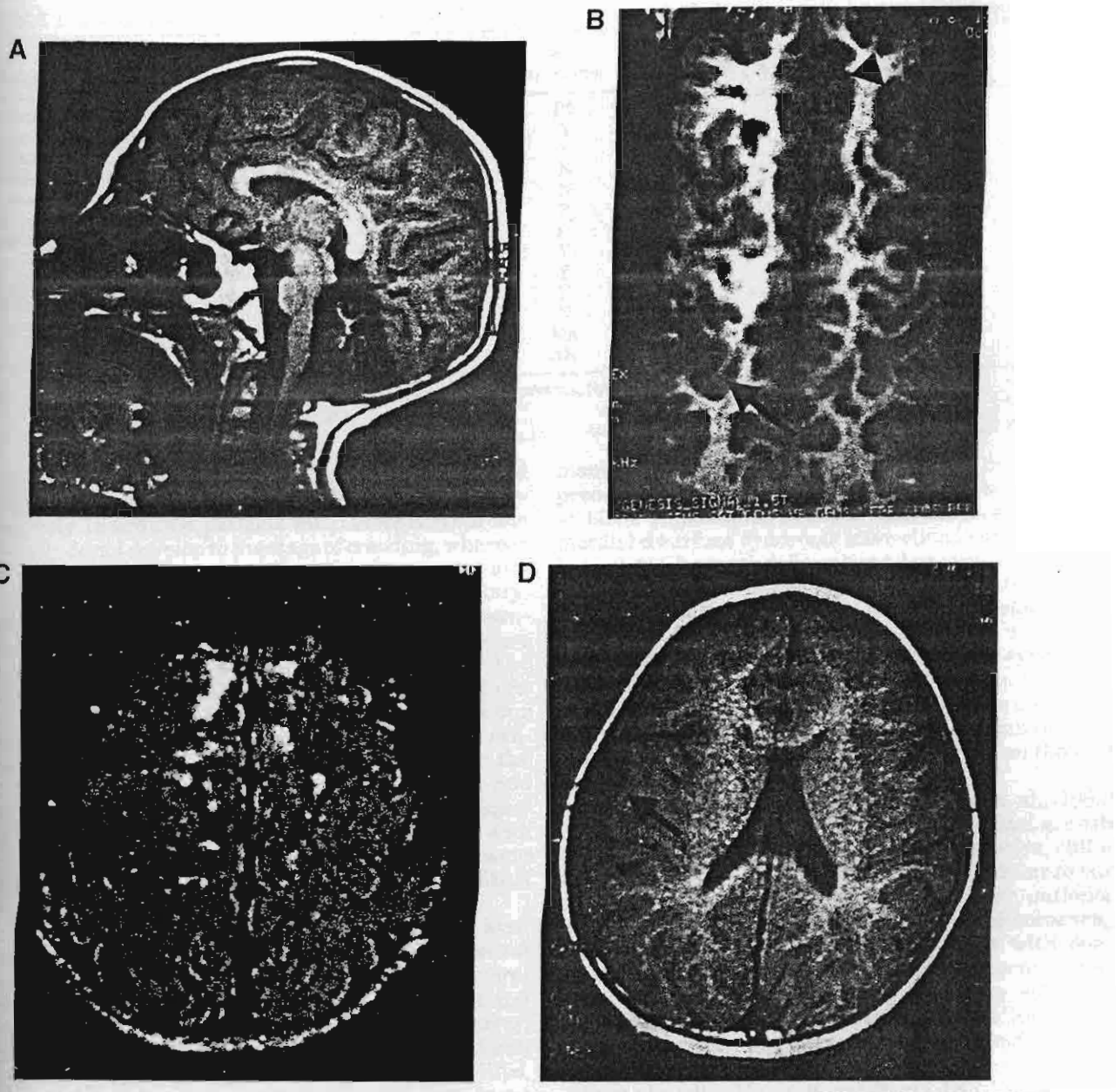

Fig. 3. Brain MRI of the elder sister (A) and the younger brother (B–D). A: Sagittal T1WI shows microcephaly and marked cerebellar atrophy with very prominent fissures between shrunken cerebellar folia and widened posterior fossa subarachnoid space. B: Reformation from volume gradient echo acquisition shows focal areas of thickened abnormal cortex with irregular "bumpy" gyral pattern in the left frontal lobe (arrow head) and right parietal lobe (arrow), suggesting cortical dysplasia. C: Axial FLAIR image shows small scattered hyperintense foci in the subcortical white matter. D: Axial T1WI shows an arachnoid cyst (arrow) in the right temporal area. Cavum septum pellucidum is also demonstrated.

joints, and severe osteoporosis. One of the children had seizures and an arachnoid cyst. We are not aware of such a combination of abnormalities seen in the sibs among known syndromes of microcephalic dwarfism with cataract and mental retardation. The fact that the two affected children were sibs without any affected family members suggests an autosomal recessive inheritance.

Syndromes from which differential diagnosis has to be made include CAMAK (CAMFAK) syndrome; COFS syndrome; microcephalic primordial dwarfism, Toriello type; Warburg micro syndrome; Martsolf syndrome; and ataxia-microcephaly-cataract (AMC) syndrome (Table I). CAMAK and CAMFAK syndromes are supposedly the same [Lowry et al., 1971; Sugarman, 1973; Scott-Emuakpor et al., 1977; Talwar and Smith, 1989]. Major features of these syndromes that are similar to those in our patients include severe mental retardation, microcephaly, cataracts, failure to thrive, and extreme osteoporosis. Patients with CAMAK syndrome are small at birth, however, and their growth is extremely slow, with one reported patient weighing

TABLE I. Clinical Manifestations of Syndromes with Microcephaly, Mental Retardation, Growth Failure, and Childhood Cataract*

Clinical findings	This report	CAMAK syndrome	COFS syndrome	Toriello syndrome	Micro syndrome	Martsolf syndrome	AMC syndrome
Growth failure	PO	PR	PR	PR	PO	PO	N
Large joint contracture	Y	Y	Y	N	Y	N	N
Osteoporosis	Y	Y	Y	N	N	Y	N
Prominent supraorbital ridge	Y	N	N	N	N	N	N
Cortical dysplasia	Y	N	N	NA	N	N	NA
Cerebellar atrophy	Y	Y	Y	NA	N	Y	NA
Spasticity	N	Y	N	N	Y	N	N
Demyelination	N	Y	Y	NA	NA	Y	NA
Kyphoscoliosis	N	Y	Y	N	Y	N	N
Hip dysplasia	N	Y	Y	N	Y	N	N
Hypogonadism	N	Y	N	NA	Y	Y	N
Abn NCV	N	Y	NA	NA	NA	N	NA
Inheritance pattern	AR	AR	AR	AR	AR	AR	AR

*AR, autosomal recessive; PO, postnatal; PR, prenatal; Y, present; N, not present; NA, information not available.

only 5,400 g at age 14 years. The sibs we reported weighed 13 and 10.5 kg at ages 9 and 6 years, respectively. In addition, patients with CAMAK syndrome typically do not develop to the stage of crawling, whereas our patients were able to walk with some support and follow commands; one of two had a limited vocabulary. In contrast to our patients, patients with CAMAK syndrome have severe spasticity, kyphoscoliosis, severe limitations of joint movement, and hip dysplasia. The facial features of the CAMAK syndrome that have been described as bird-like are markedly different from our patients. Neurologically, patients with CAMAK syndrome show a major defect in myelination both in the central and peripheral nervous systems [Talwar and Smith, 1989], whereas our patients had cortical dysplasia and cerebellar atrophy as the prominent feature with no evidence of demyelination. An arachnoid cyst present in one of our patients has not been reported in CAMAK syndrome.

Similar to our patients, patients with COFS syndrome have growth failure, microcephaly, severe mental retardation, cataracts, prominent nose, large ears, progressive joint contractures, and osteoporosis [Pena and Shokeir, 1974]. In contrast to our patients, however, patients with COFS syndrome have microphthalmia, deep set eyes, blepharophimosis, overhanging lips, scoliosis, hip dysplasia, camptodactyly, prominent heels, posteriorly placed second metatarsal, hypotonia, difficulty feedings, recurrent pulmonary infections, and delayed teeth eruption. In addition, patients with COFS syndrome typically have bilateral intracranial calcifications in the region of the basal ganglia, ventriculomegaly, demyelination, agenesis of corpus callosum, cortical atrophy, cerebellar atrophy, and optic nerve atrophy [Linna et al., 1982; Del Bigio et al., 1997; Meira et al., 2000]. Of these symptoms, our patients had only cerebellar atrophy. Prominent supraorbital ridges, prognathism, cortical dysplasia, and cavum septum pellucidum were present in our patients but not in patients with COFS syndrome.

As with our patients, patients with microcephalic primordial dwarfism, Toriello type [Toriello et al., 1986] have growth deficiency, microcephaly, cataracts, and mental retardation. The onset of growth deficiency is prenatal, however, whereas our patients were normal at birth. In addition, patients with microcephalic primordial dwarfism syndrome have clinodactyly, enamel hypoplasia, immune deficiency and generalized delay of ossification, which were not present in our patients. The limitations of joint movement and severe osteoporosis present in our patients has not been reported in patients with the microcephalic primordial dwarfism syndrome. Importantly, the facial features of patients with the microcephalic primordial dwarfism syndrome are receding forehead, downslanting palpebral fissures, and micrognathia that are markedly different from those of this study.

In Warburg micro syndrome [Warburg et al., 1993; Megarbane et al., 1999], signs include postnatal growth failure, microcephaly, severe mental retardation, childhood cataracts, and mild contracture as is similar to our patients. In contrast to our patients, however, patients with Warburg micro syndrome have microcornea, borderline micro-ophthalmus, small pupils with posterior synechiae, optic nerve atrophy, hypogenitalism, hypertrichosis, kyphosis, and spastic palsy with hip dislocation as prominent features. In addition, our patients had severe osteoporosis, which is not present in patients with Warburg micro syndrome.

Patients with Martsolf syndrome [Harbord et al., 1989] may have microcephaly, mental retardation, cataracts, postnatal growth failure, osteoporosis and seizures. In contrast to our patients, however, patients with Martsolf syndrome have hypogonadism, cardiomyopathy, marked hypotonia with exaggerated tendon reflexes, lax finger joints, lumbar lordosis and generalized cerebral atrophy and delayed myelination. Contractures were noted in our patients but not with Martsolf syndrome.

Patients with AMC syndrome [Ziv et al., 1992] have mental retardation, microcephaly, and cataracts. In contrast to our patients, however, patients with AMC syndrome have ataxia, hypotonia, and nystagmus as major features but do not show growth retardation, joint contractures, or osteoporosis. Psychomotor retardation is present in only one of three reported AMC patients.

In 1987, Bouwes Bavinck et al. [1987] reported a mother and her son with microcephaly, eye anomalies, short stature, and mental deficiency. Unlike our patients, the son had ptosis, blepharophimosis, low-set ears, hydroureters, hydronephrosis, cryptorchidism, and hyperextensibility of fingers and toes. The mother had several eye abnormalities including iris and choroidal colobomata, microphthalmia, microcornea, and no light perception-vision. In addition, they were only mildly mentally delayed. Patients with carbohydrate deficient glycoprotein syndrome have cerebellar atrophy [Grunewald and Matthijs, 2000]. The possibility of these two children having the classic form of the syndrome, however, has been ruled out by the normal transferrin analysis.

CONCLUSION

In summary, we report on two sibs with a previously undescribed autosomal recessive syndrome comprising postnatal-onset growth deficiency, microcephaly, mental retardation, cataract, prominent supraorbital ridge, large joint contractures, severe osteoporosis, cortical dysplasia and cerebellar atrophy.

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บทความที่ 5

Clinical, Pathological, and Electron Microscopic Findings in Two Thai Children with Pompe Disease

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Abstract

The authors report on a Thai boy who first presented at age 7 months and an unrelated Thai girl in her neonatal period with hypotonia, cardiomegaly and hepatomegaly. Their chest roentgenograms showed markedly enlarged hearts, EKGs showed abnormally shortened PR intervals with gigantic QRS complexes, and electron microscopic studies of their skin samples showed glycogen accumulations surrounded by membranes. The boy died at age 22 months and the girl at age 9 months due mainly to cardiorespiratory failure. Autopsy of the girl showed marked accumulation of glycogen in the liver, heart and numerous additional tissues including her brain. The clinical, pathological, and electron microscopic findings of these two children are consistent with the diagnosis of Pompe disease.

Pompe disease is an autosomal recessive disorder of glycogen metabolism resulting from deficiencies in activity of the lysosomal acid α-glucosidase. Definite diagnosis of the disease can be made from a biochemical test or a mutation analysis. To the authors' knowledge, no service laboratories in Thailand offer the tests. Because Thai children have occasionally been reported to be affected by Pompe disease, an attempt to establish a definite diagnostic test for Pompe disease in Thailand should be encouraged. With a definite diagnosis, the proper genetic counseling and prenatal diagnosis could be offered to the families.

Key word : Glycogen Storage Disease, Pompe Disease, Electron Microscopy

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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.

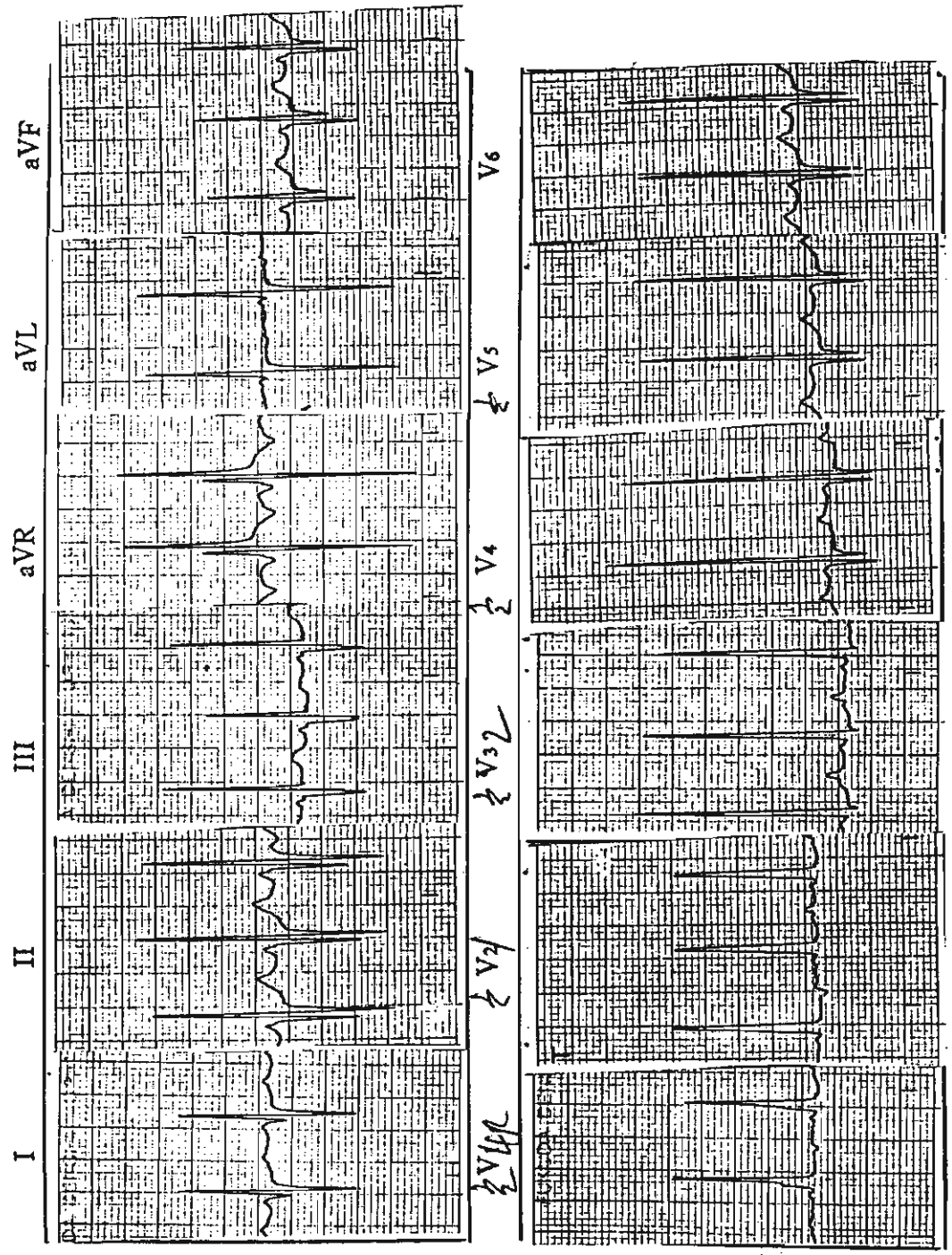

Fig. 1. The electrocardiogram of Patient 1 shows a short PR interval with large QRS complexes.

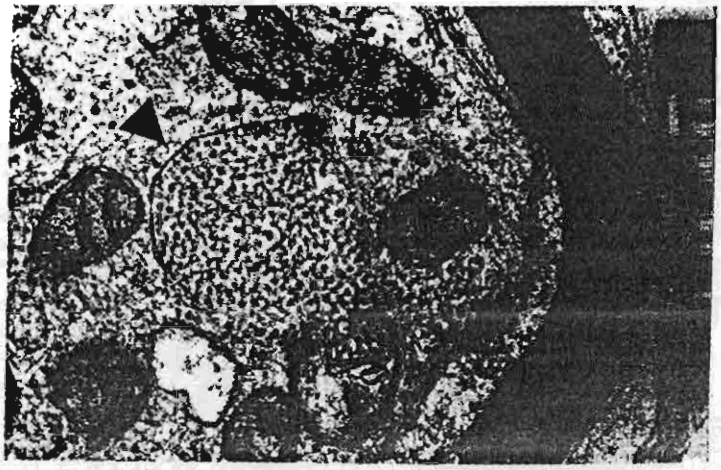

Fig. 2. Electron microscopic findings of patient 1. Note an accumulation of glycogen particles surrounded by a single membrane (arrow head).

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].

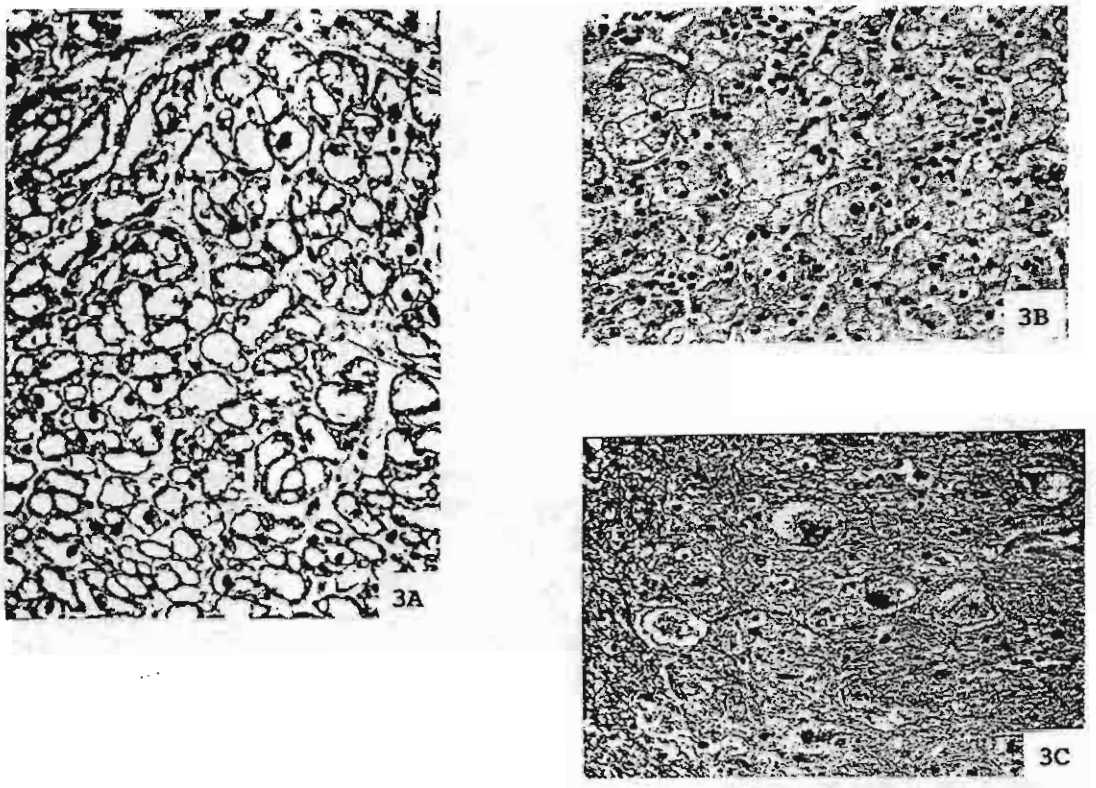

Fig. 3. Pathology of Patient 2. All of the cardiac muscle cells contain intracytoplasmic round to oval clear space (A). The liver cells are enlarged with multiple cytoplasmic vacuoles (B). Neurons in the dentate nucleus of the cerebellum are distended with cytoplasmic foamy substance, displacing the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

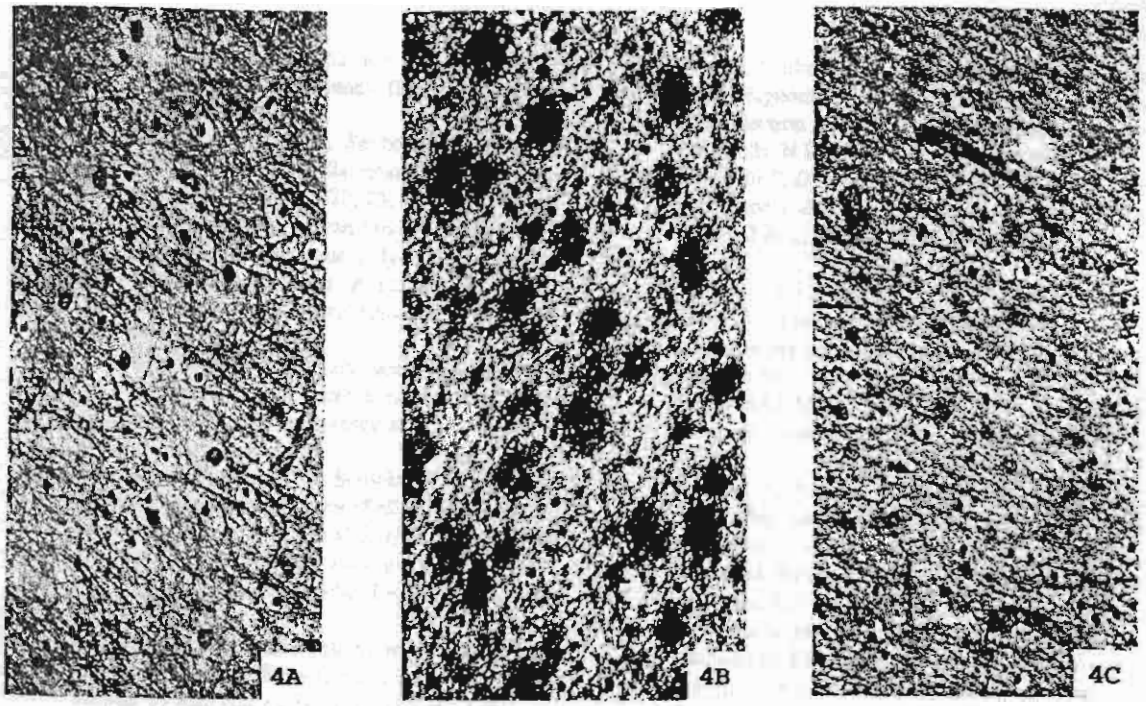

Fig. 4. Pathology of Patient 2. Astrocytes in the cerebral white matter are enlarged with foamy cytoplasm (A), which is PAS (periodic acid Schiff)-positive (B) diastase-liable (C), characteristic of glycogen (A, H&E; B, PAS; C, PAS with diastase pretreatment).

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

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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.

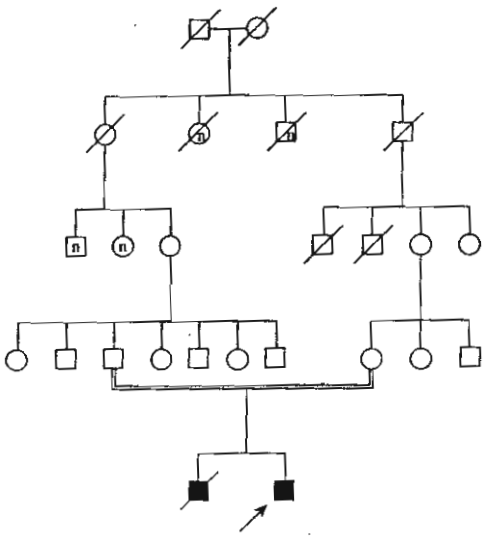

Fig 1–Pedigree of family 1.

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

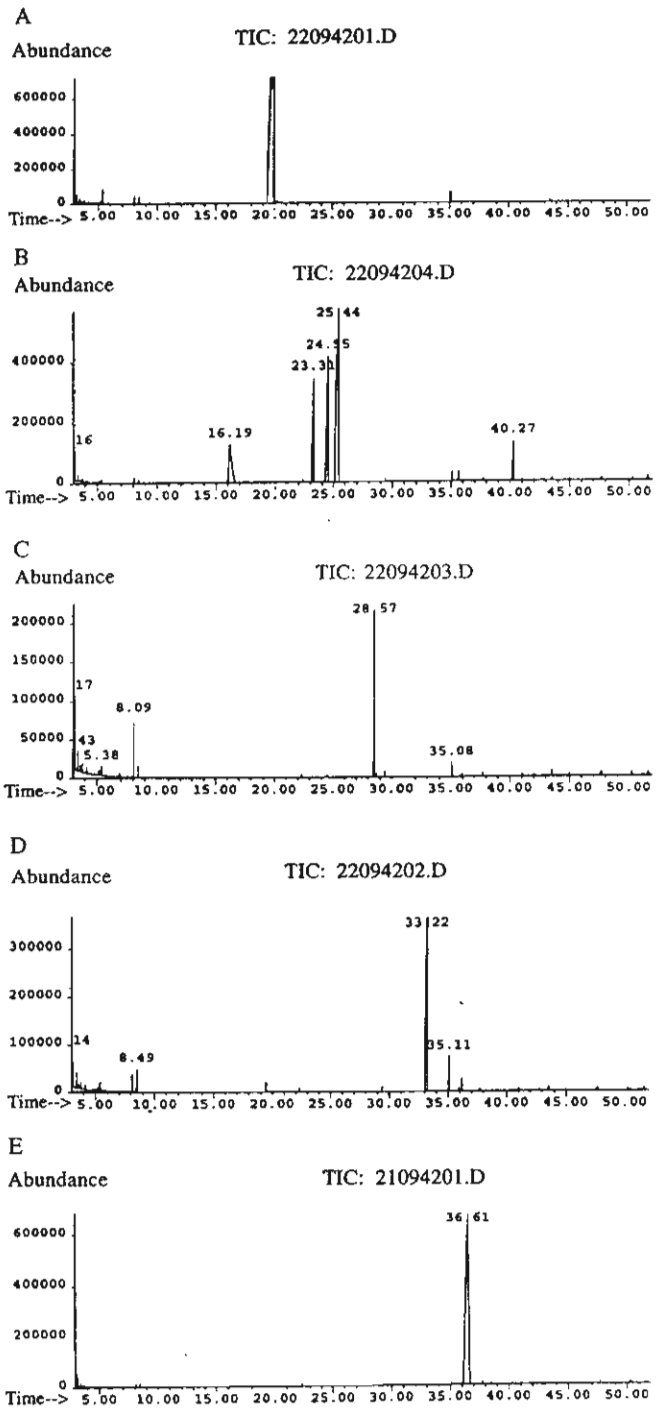

Fig 2–tracings of adipic acid (A), succinyl acetone (B), orotic acid (C), sebacic acid (D), and undecanedioic acid (E) as internal standard.

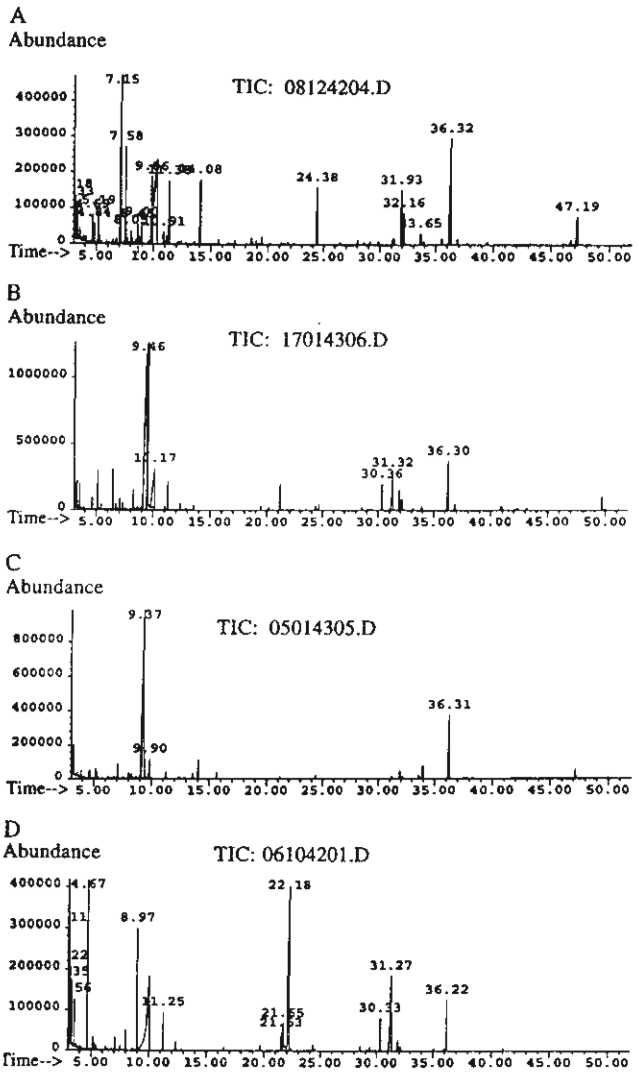

Fig 3–Urine organic acid tracing of patient 1 (A), patient 3 (B), patient 5 (C), and patient 7 (D). Panel A showed large peaks of 3-hydroxypropionate at 7.15 minutes and methylcitrate at 31.93 and 32.16 minutes. A small peak of 3-OH isovalerate was present at 9.00 minutes. The peak at 36.32 minutes was 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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We would like to express our gratitude to Dr George Thomas of the Kennedy Kreiger Institute, and Dr Tina Cowan of The University of Marland, Baltimore, MD, USA for kindly provided us with the organic acid libraries. We are also grateful to Dr Voranit Kongmebhol, Dr Nat Tansrisawad and Ms Vullada Puranaveja of the Department of Forensics Medicine, Chulalongkorn University, Thailand for their cooperation.

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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-

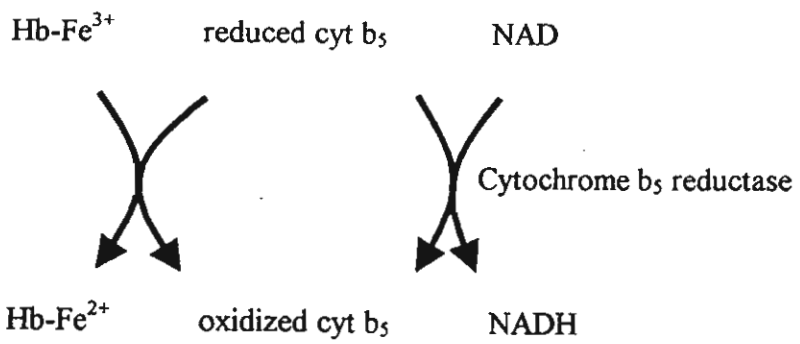

Fig. 1. Erythrocyte pathways for reduction of methemoglobin.

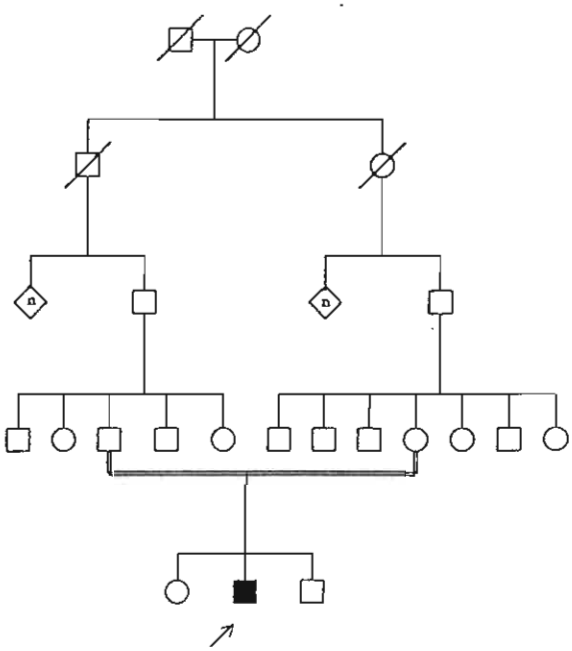

Fig. 2. Pedigree.

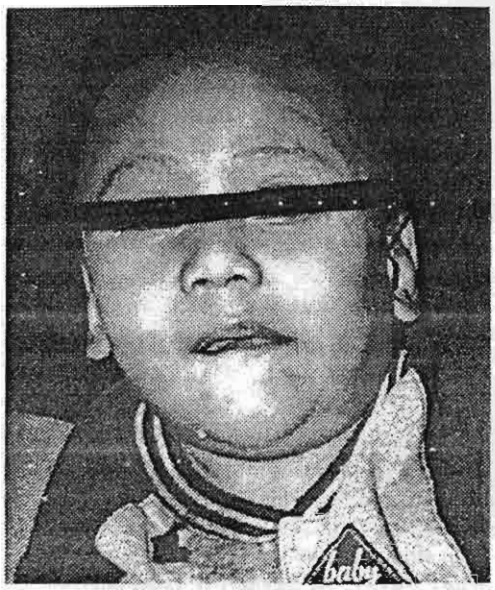

Fig. 3. The patient's face.

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.

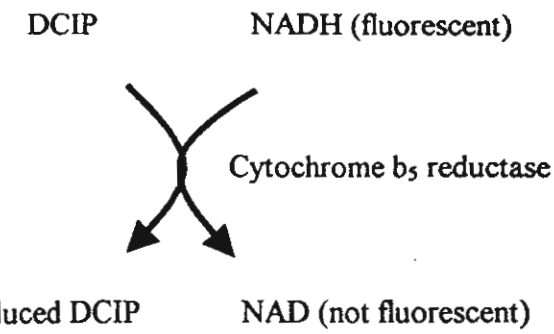

Fig. 4. Principle of the qualitative assay of the activity of the cytochrome b_5 reductase. After adding whole blood to a hemolyzing agent, NADH, and DCIP, in the presence of cytochrome b_5 reductase in the red cells, the DCIP is reduced by NADH. During the reaction, NADH, which fluoresces when illuminated 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.

ACKNOWLEDGMENT

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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	

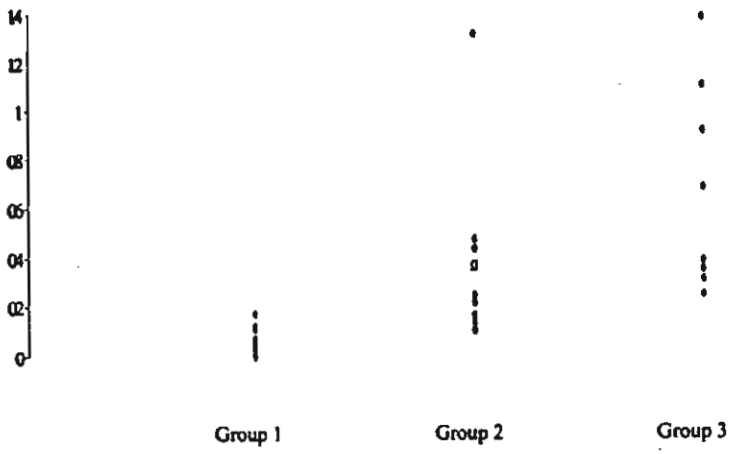

Fig. 1. The basal LH/FSH ratio in 3 groups.

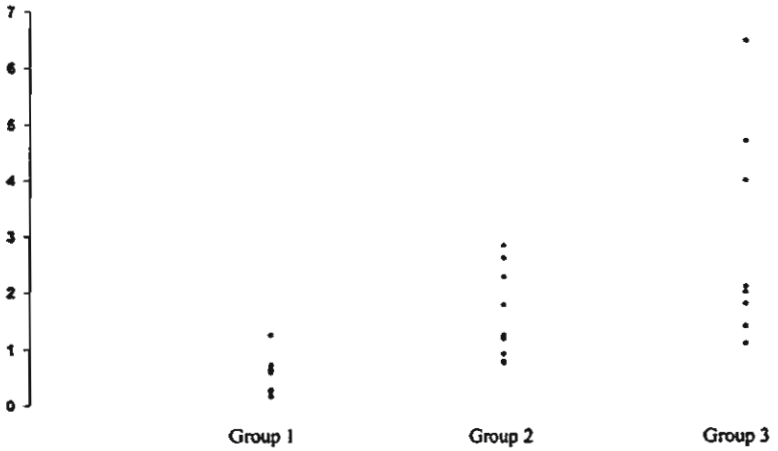

Fig. 2. The peak LH/FSH ratio in 3 groups.

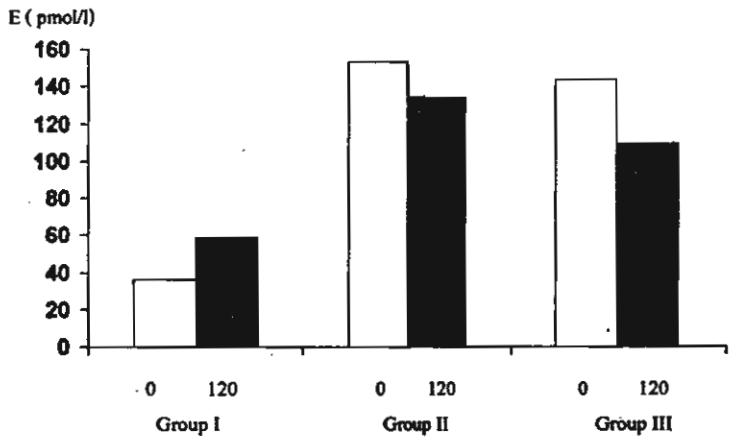

Fig. 3. Estradiol (E) at 0 and 120 min in 3 groups.

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

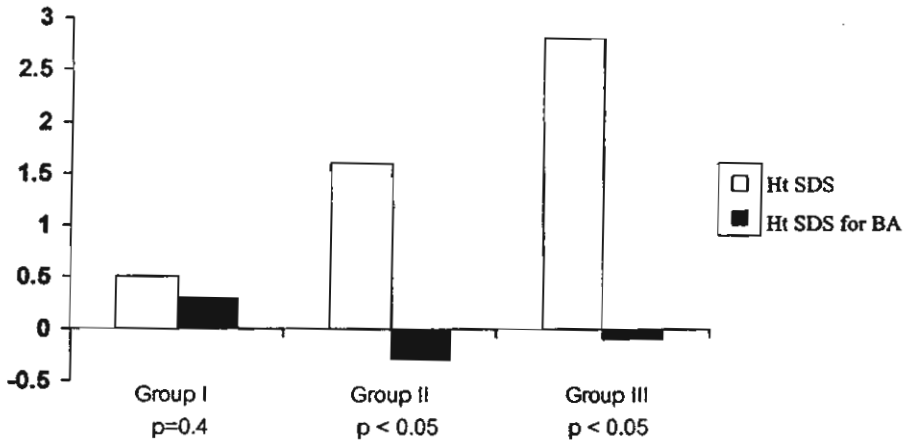

Fig. 4. Ht SDS and Ht SDS for BA in 3 groups.

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

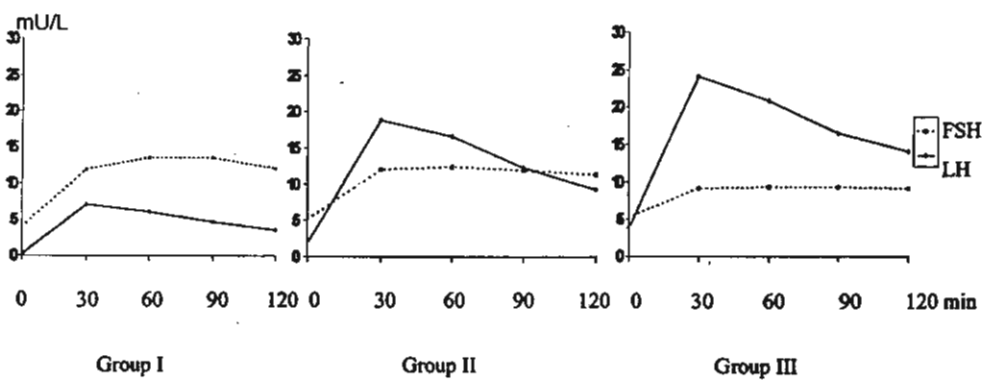

Fig. 5. FSH and LH during GnRH test in 3 groups.

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
จดหมายเหตุทางแพทย์ ฯ 2543; 83: 1105–1111

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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-

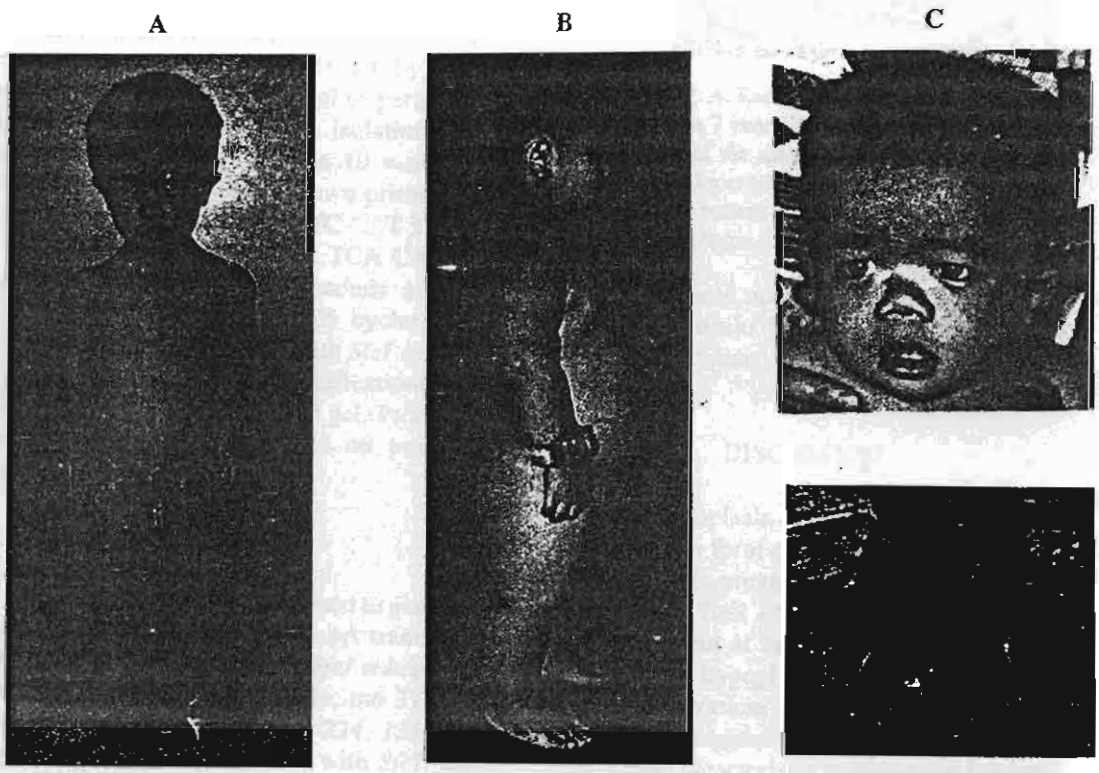

Fig 1–Clinical features. A. Patient 1 at 7 years of age showing disproportionate short stature with rhizomelia. B. Patient 2 at 6 years old revealing frontal bossing, overweight, and lumbar lordosis. C. Patient 3 at 11 months old showing maxillary hypoplasia (upper panel) and a trident hand (lower panel).

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

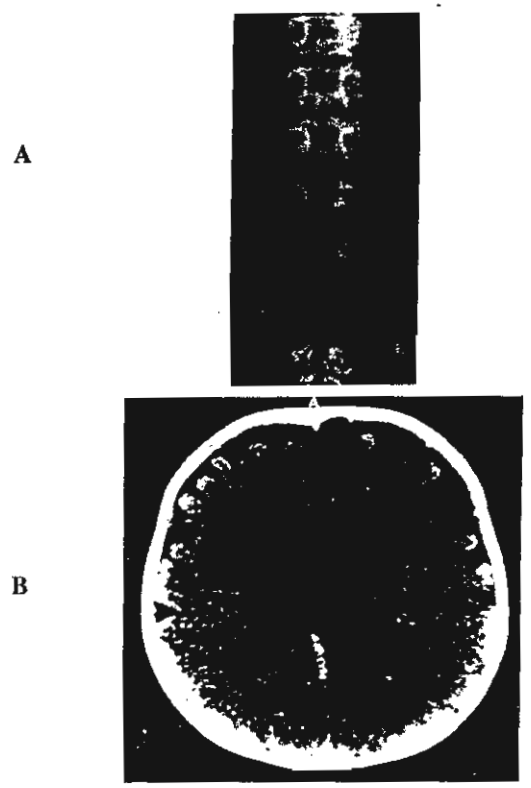

Fig 2–Imaging. A. Radiograph of lumbar spine of patient 1 at 7 years of age revealing caudal narrowing of the spinal canal and a shadow of a ventriculoperitoneal shunt. B. CT scan of the brain of patient 3 at 11 months old revealing hydrocephalus.

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

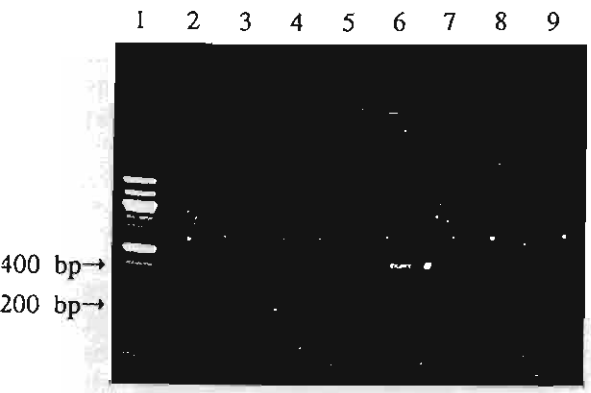

Fig 3–Restriction enzyme detection of the G380R mutation in achondroplasia. Lane 1 represents a 100 bp marker with the bands at 200 and 400 bp indicated with arrows. Lanes 2 and 3 were of the mother of patient 1; lanes 4 and 5 patient 1; lanes 6 and 7 patient 3; lanes 8 and 9 the mother of patient 3. 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

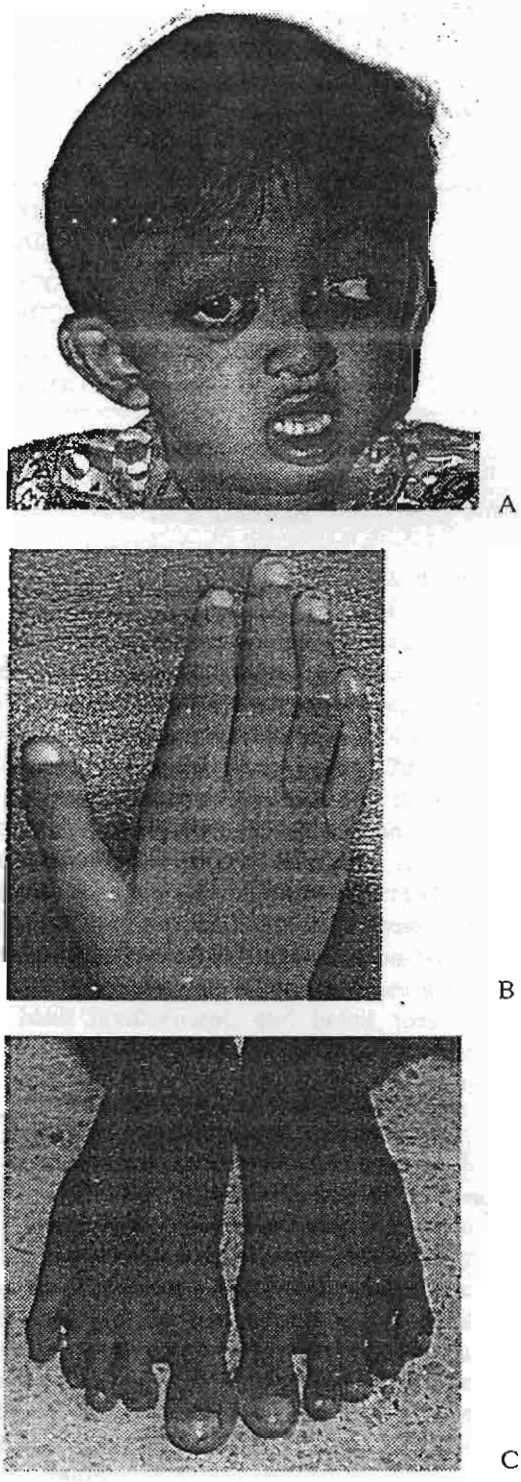

Fig 1–A. Face B. Right hand C. Feet of the patient.

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).

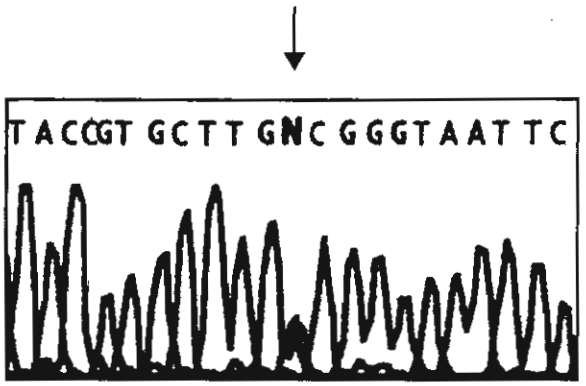

Fig 2–The backward strand sequence of the *FGFR2* exon 10 revealed a G->C transversion (indicated in the figure by an arrow).

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

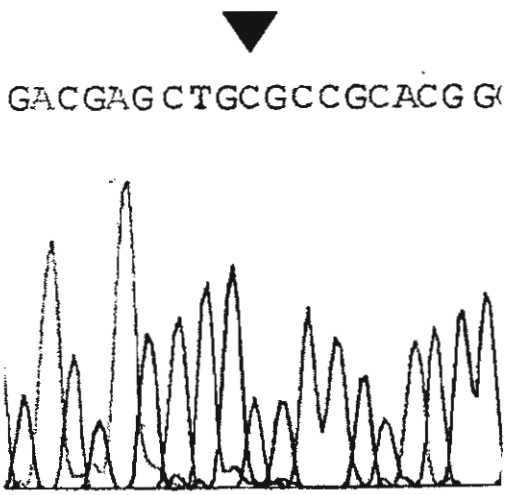

Fig. 1. The sense sequence electropherograms of the plasmid insert of the proband. The arrow head indicates the substitution of a C for the normal T.

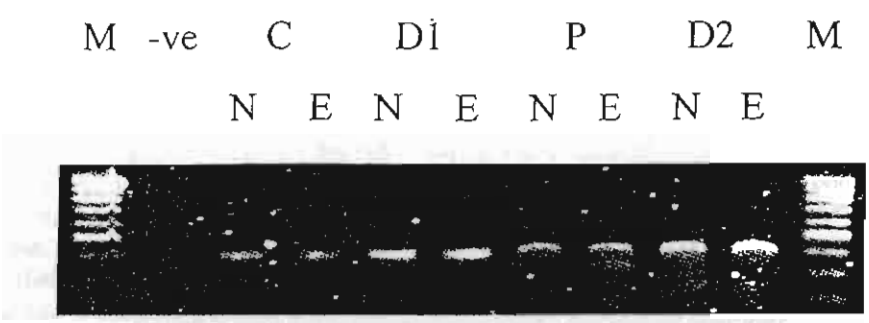

Fig. 2. Restriction enzyme detection of the C634R mutation in MEN 2A. Lanes 1 and 11 represents a marker (M). Lane 2 serves as a negative control (-ve). Lanes 3 and 4 were of a normal control (C); lanes 5 and 6 the proband's elder daughter (D1); lanes 7 and 8 the proband; lanes 9 and 10 the proband's younger daughter (D2). Lanes 3, 5, 7, and 9 were PCR products without adding restriction enzymes (N) and only the undigested 384 bp bands were presented. Lanes 4, 6, 8, and 10 were PCR products mixed with restriction endonuclease enzyme *Hha* I (E). The new smaller band in lane 8 demonstrates that the proband is heterozygous for the 1900T>C mutation.

Fig. 3A. ^{131}I MIBG shows abnormal accumulation of tracer at both adrenal glands, more on the right side (thin arrows).

Fig. 3B. MRI demonstrates well-marginated mixed solid and partly cystic bilateral adrenal masses measuring 10 x 5 x 5.5 cm and 6 x 4 x 3 cm right and left adrenal respectively (thick arrows).

clones showed the mutated sequence (Fig. 1), while the other was normal (data not shown). Restriction enzyme digestion of the patient and her two daughters revealed the pattern of mutation only in the proband (Fig. 2). The mutation is expected to result in subsequent substitution of an arginine for the normal cysteine at codon 634 (C634R).

DISCUSSION

The presented patient had medullary thyroid carcinoma, pheochromocytoma and primary hyperparathyroidism, which are typical manifestations of MEN 2A. She was previously described but not molecularly characterized[5]. No mutation analysis was performed either in the other reported Thai patient

with MEN 2A[6].

MEN 2A is transmitted in an autosomal dominant manner with virtually 100 per cent penetrance [9]. The first tumor is usually medullary thyroid carcinoma, which may occur very early in life[10]. Its only potentially curative treatment is surgical removal of all thyroid tissue, a goal not commonly achieved in patients with clinically manifest carcinoma. The detection of mutations in the *RET* gene has important diagnostic and therapeutic impacts. Genetic testing of family members at risk allows one to identify individuals carrying mutant alleles with very high specificity and sensitivity[11]. Subsequently, total thyroidectomy can be performed in a prophylactic attempt. Currently, the procedure is recommended at 5 years of age[12,13]. Individuals who carry the mutation are also subjected to clinical and laboratory surveillance for pheochromocytoma and primary hyperparathyroidism.

The authors developed a DNA test to identify a mutation in a Thai woman with MEN 2A. She was found to be heterozygous for 1900T>C (C634R). The C634R mutation is one of the most frequent changes found in patients with MEN 2A[14]. It is a gain-of function mutation[15]. In addition, it is associated with the earliest development of the thyroid carcinoma compared to mutations at codons 618, 620, and 804[16]. The presented patient has two daughters. If either of them carried the mutation, total thyroidectomy would have been recommended. Fortunately, only normal sequence was found after their blood samples were tested for the mutation.

In summary, the authors developed a genetic test for patients with MEN 2A in Thailand, making accurate DNA-based diagnosis of their at-risk family members possible before development of the disease. Identification of individuals carrying mutant alleles has an important therapeutic impact.

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การกลายพันธุ์ชนิด C634R ในจีนส์ RET ในหญิงไทยที่เป็น Multiple endocrine neoplasia type 2A

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Multiple endocrine neoplasia type 2A (MEN 2A) เป็นโรคที่ถ่ายทอดแบบจีนส์เด่นบนออโตโซม มีลักษณะสำคัญคือ medullary thyroid carcinoma, pheochromocytoma และ primary hyperparathyroidism โดยมะเร็งชนิดที่เกิดขึ้นก่อน มักเป็น medullary thyroid carcinoma โรคนี้เกิดจากการกลายพันธุ์ในจีนส์ RET การตรวจการกลายพันธุ์มีประโยชน์ทั้งในด้านการวินิจฉัยและการรักษา วิธีดังกล่าวสามารถใช้วินิจฉัยผู้ที่มีการกลายพันธุ์ได้อย่างแม่นยำ ซึ่งผู้ที่พบว่ามีการกลายพันธุ์ควรได้รับคำแนะนำให้ตัดต่อมไทรอยด์ออกทั้งหมดก่อนอายุ 5 ปี เพื่อป้องกันการเกิด medullary thyroid carcinoma

คณะผู้วิจัยได้พัฒนาวิธีการวิเคราะห์หาการกลายพันธุ์ในผู้ป่วยหญิงไทยรายหนึ่งที่เป็น MEN 2A และพบว่าผู้ป่วยมีการกลายพันธุ์ชนิด heterozygous 1900T>C (C634R) โดยบุตรสาวทั้งสองของผู้ป่วยไม่มีการกลายพันธุ์ดังกล่าว

การพัฒนาการตรวจหาการกลายพันธุ์ในจีนส์ RET สำหรับผู้ป่วย MEN 2A ในประเทศไทย ทำให้สามารถใช้วิธีการดังกล่าวมาวินิจฉัยสมาชิกในครอบครัวของผู้ป่วยได้อย่างแม่นยำ ซึ่งจะมีผลในด้านการป้องกันและรักษาต่อไป

คำสำคัญ : มัลติเปิ้ล เอนโดครีน นีโอเพลเซีย, อาร์อีที, การตรวจหาการกลายพันธุ์

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บทความที่ 12

ystinuria : Cause of Recurrent Renal Stones in a 4-ear-Old Girl

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Abstract

This paper presents the case report of a 4-year and 6-month old girl with cystinuria. She clinically presented with recurrent radiopaque renal stones since the age of 3 years. She received 2 subsequent operations of pyelolithotomy combined with ureterolithotomy at the age of 3 years 6 months, and pyelolithotomy alone at the age of 5 years. She was initially diagnosed as having cystinuria by the presence of hexagonal plate crystals in her acidified urine and positive for the urinary cyanide-nitroprusside test. The diagnosis was confirmed by urinary amino acid analysis using quantitative ion-exchange chromatography which revealed increased amounts of cystine and dibasic amino acids of lysine and ornithine. In spite of maintaining a high fluid intake and alkalinizing urine by giving potassium citrate after the first operation, recurrent renal stones were found. Therefore, after the second operation, D-penicillamine was additionally introduced. During the 18-month follow-up, although there were recurrent renal stones, the rate of stone formation was slower. To the authors' knowledge, this is the first case report in Thailand.

Key word : Recurrent Renal Stones, Cystinuria, D-Penicillamine, Alkalinization

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Cystinuria is an autosomal recessive genetic defect of transepithelial transport of cystine and the other dibasic amino acids in the kidney and intestine (1,2). The renal transport defect is expressed by the excessive urinary excretion of cystine, the least soluble amino acids, which results in cystine crystallization and subsequent formation of a cystine stone. Cystinuria is the cause of 1 per cent to 2 per cent of renal stones observed in adults(3,4) and about 6 per cent to 8 per cent of pediatric urinary calculi in Western countries(5,6). As the genetic transport defect exists since birth, stone formation begins in the first decade of life and continues life long. The majority of patients with cystinuria will suffer recurrent renal stone disease during their lifetime(7) with subsequent urinary tract obstruction, infection and possible renal insufficiency(7). Cystine stones are poorly fragmented by extracorporeal shock wave lithotripsy (ESWL) and hence operative lithotomy is often necessary. To prevent recurrent renal stone formation, regular medical treatment is of particular importance in affected patients(8).

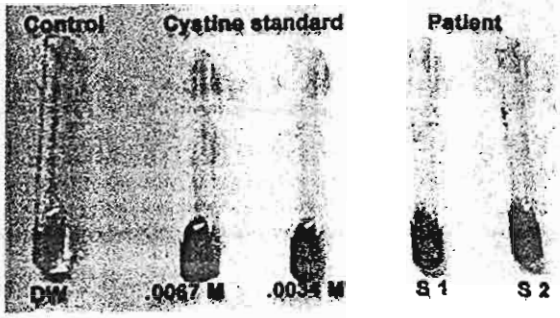

Fig. 1. The urine cyanide-nitroprusside test of the patient (S_1 and S_2) revealed magenta color compared with cystine standard and control using distilled water.

CASE REPORT

A 4-year and 6-month-old girl was referred to the Pediatric Nephrology Unit, Ramathibodi Hospital for the management of recurrent renal stones. Her past medical history included recurrent abdominal pain and urinary tract infections at the age of 3 years. Her intravenous pyelography revealed bilateral hydronephrosis, right radiopaque renal stones and left distal ureteric stones. Right pyelolithotomy and lower left ureterolithotomy were successfully performed at the age of 3 years and 6 months. She had been doing well since the calculi were removed. One year post-operatively, she developed recurrent bilateral renal stones. Ultrasonography showed bilateral hydronephrosis, 2 small stones in the right upper and middle calices and a large one in the lower calyx, about 2.2 cm in diameter and a left lower pole renal stone, about 0.8 cm.

On physical examination, the patient was a healthy-looking child in no acute distress. The only abnormal finding was surgical scars on the right flank area and left lower abdomen. Laboratory studies revealed normal complete blood count ; blood urea nitrogen 11 mg/dl, serum creatinine 0.5 mg/dl, sodium 138 mmol/L, potassium 4.53 mmol/L, chloride 110 mmol/L, total CO_2 20.7 mmol/L, calcium 10.0 mg/dl, phosphate 4.8 mg/dl and uric acid 4.5 mg/dl. Urinalysis revealed yellowish color, pH 5, specific gravity 1.020, markedly positive blood, WBC 5-10/HPF, RBC >100/HPF, no casts and few hexagonal crystals. The 24-hour urine calcium was 0.19 mg/kg/day. The cystine test was performed on the patients fresh and first-morning-voided urine using cyanide-nitroprusside. After adding sodium cyanide and nitroprusside, a purple red or magenta color was revealed which was suggestive of the presence of cystine (Fig. 1). Subsequently, urinary amino acid analysis by quantitative ion-exchange chromatography revealed increased amounts of cystine and dibasic amino acids of lysine and ornithine (Table 1).

Her mother had also had a right ureteric stone which was removed at the age of 26 years. She has been doing well and no recurrence of renal stone was found during the 9-year follow-up. There was no family history of consanguinity. No other family member had history of renal stones. Laboratory findings for her mother including complete blood counts, serum electrolyte, blood urea nitrogen, serum creatinine, calcium, uric acid and urinalysis were within normal limits. The 24-hour urine calcium was 73 mg/day. Her urine cyanide-nitroprusside test was negative for cystine. There was no radiopaque stone on a recent abdominal radiograph. The urinary amino acid analysis of the mother revealed 25 per cent

Table 1. The urinary amino acid analysis of the patient and her mother by the quantitative ion exchange chromatography.

Amino acids	Normal range in children[a] (mg/g creatinine)	Patient's values[b]	Normal mean ± SD in adults (mg/g creatinine)	Mother's values[c]
Cystine	4.3-48.0	286.5	16.5 ± 6.9	30.0
Ornithine	4.6-44.3	440.1	1.3 ± 0.5	2.2
Lysine	10.4-184.5	549.7	21.0 ± 17.2	3.3
Arginine	1.5-110.0	5.3	1.7 ± 0.7	2.7

a : N = 13, 1-13 years
b : All urine amino acids except cystine and dibasic amino acids were within normal limits
c : All urine amino acids except cystine were within normal limits

:reased amounts of cystine but amounts of dibasic iino acids were within normal limits compared with : control (Table 1).

At the age of 4 years and 6 months after 'ealing recurrent renal stones, the patient had been :ouraged to maintain a high fluid intake and a v salt diet. Potassium citrate had been given for :alinizing urine in order to increase cystine solu- ity. Her serial urine specific gravity ranged from)10 to 1.015 and urinary pH invariably exceeds 7.0. e had been doing well without abdominal pain and nary tract infection. Six months after receiving ne preventive treatment, repeated abdominal ultra- nography revealed an increase of the stone size m 0.8 cm to 1.3 cm in the left kidney and a large ne 2.2 cm in the right kidney. Moderate right dronephrosis and mild left hydronephrosis were idenced. She underwent the second operation of ht pyelolithotomy, at the age of 5 years. Stone alysis proved cystine stone. Because the high resis and urine alkalinization failed to slow down cystine stone formation, D-penicillamine was led after the second operation in order to increase stine solubility. One year later, at the age of 6 rs, repeated abdominal ultrasonography showed d hydronephrosis of the right kidney and no hydro- hrosis of the left kidney. There were two new all stones at the lower pole of the right kidney, asuring about 0.3 cm. Fortunately, the left renal ne of 1.3 cm had decreased to 0.3 cm diameter.

At her last visit when she was 6 years and nonths old, the recent abdominal ultrasonography wed the same size of multiple stones in both neys measuring 0.3 cm without obstruction. Her ent serum creatinine was 0.6 mg/dl. She had done y well in the elementary school with medical pre- tive treatment for cystine stone.

DISCUSSION

Cystinuria is not an uncommon cause of pediatric renal stones reported from Western countries[5,6]. Cystine stones are particularly prevalent in the second or third decade of life but occur infrequently in infancy[9]. In Thailand, the prevalence of pediatric renal stone is unknown and cystinuria is a rare cause in childhood. Because cystine stone is moderately radiopaque secondary to the presence of the sulfur atom in the molecule, it may be misdiagnosed as being a calcium containing stone which delays the diagnosis of cystinuria. The presented patient developed bilateral renal cystine stones in first few years of life with subsequent obstruction and recurrent urinary tract infections requiring right pyelolithotomy and left ureterolithotomy at the age of 3 years and 6 months. Due to the undiagnosed cause of her renal stones and lack of medical preventive treatment for cystine stones, she had recurrent stone formation within 1 year after the first operation.

Urinary excretion of cystine in a normal individual is less than 20 mg/g creatinine. Patients of homozygous cystinuria excrete more than 400 mg/day or more than 250 mg/g creatinine[8,10]. The major lithogenic factor in patients with cystinuria is the high concentration of cystine in the urine, because of its relative insolubility, crystallizes at the usual urine pH (5.5-6.5). Dent and Senior reported that the solubility of cystine in the urine sharply rises with higher pH, up to 500 mg/L in urine pH above 7.5[11]. The presence of hexagonal cystine crystals in fresh and first-morning-voided urine may be a pathognomonic for cystinuria but this is seen only in a minority of cases[12,13]. In the presented patient, hexagonal crystals of cystine were not found in repeated urinalysis until her urine was concentrated and pH was low to 5.0. Also, the positive for urinary cyanide-

nitroprusside test is suggestive of the presence of cystine excretion exceeding 75 mg/L[9,14] which is a rapid and simple screening test for patients with cystinuria. It may also detect asymptomatic patients and some patients with heterozygous cystinuria[15]. The presence of urinary hexagonal crystals and the positive cyanide-nitroprusside test indicate the presumptive diagnosis of cystinuria. Her subsequent urinary amino acid analysis by the quantitative ion exchange chromatography documenting elevated levels of cystine, 286.5 mg/g creatinine and dibasic amino acids of lysine and ornithine confirms the diagnosis of cystinuria.

Cystinuria is an amino acid transport disorder, transmitted as an autosomal recessive inheritance[1]. Rosenberg et al[16] described 3 types of classic cystinuria of obligate heterozygotes in the proband's family according to the urinary phenotype. Type I heterozygotes shows normal aminoaciduria, whereas type II and III heterozygotes show high or moderate excretion of cystine and dibasic amino acids, respectively. Heterozygotes for cystinuria generally do not excrete enough cystine into the urine to be at increased risk for cystine calculi, but they may be at increase risk for calcium oxalate stone formation when compared with normal control groups [17,18]. In the present report, the patient's mother had negative results of cyanide-nitroprusside test and her urinary amino acid chromatography showed only mild elevation of cystine but normal excretion of dibasic amino acids. The finding is consistent with type I heterozygotes of cystinuria. She also had experience of a ureteric stone which has not recurred.

The objective of medical treatment is to reduce the urinary cystine concentration below its solubility limit which would prevent recurrent cystine stone formation. Manipulations of urinary pH to optimal alkalinity and maintenance of adequate urine volumes to prevent cystine crystallization represent realistic and pragmatic means of cystine stone chemoprevention[4,7-9]. The goal of alkali therapy by means of sodium citrate and/or potassium citrate is to maintain urine pH up to 7.5[8]. Due to the sodium-induced enhancement of urinary calcium and cystine excretion, potassium citrate is preferable[8-9, 19]. After the first operation, the presented patient has been encouraged to maintain high fluid intake and high diuresis and has taken potassium citrate for urine alkalinization since the presumptive diagnosis of cystinuria. Her urine specific gravity has been maintained at 1.010 to 1.015 and urinary pH up to 7.5-8.0. However, the above treatments were not sufficient enough for dissolving pre-existing stones or preventing new stone formation. Therefore, after the second operation of right pyelolithotomy, additional therapy of D-penicillamine was initiated. D-penicillamine is a cystine-binding agent composed of a thial group which is able to bind the sulfide moiety of cystine. D-penicillamine-cystine complex is 50 times more soluble than cystine itself[20]. However, after 18 months of D-penicillamine administration, the patient still had new stones but the rate of stone formation is much slower and the dissolution of the pre-existing stone was also evidence.

In summary, cystinuria is a rare cause of renal stone in early childhood. If the recurrent radiopaque renal stones are found soon after the operation, the diagnosis of cystinuria should be suspected. Screening for cystinuria by looking for urinary hexagonal crystals and performing the cyanide-nitroprusside test is suggested. Medical intervention in patients with cystinuria is essential not only for preventing new stone formation but also protecting renal impairment.

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ซิสตินยูเรีย : สาเหตุของการเกิดนิ่วที่ไตชนิดเป็นซ้ำในเด็กหญิงไทยอายุ 4 ปี

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รายงานผู้ป่วยเด็กโรคซิสตินยูเรีย อายุ 4 ปี 6 เดือน ผู้ป่วยได้รับการตรวจพบว่ามีก้อนนิ่วที่ไตตั้งแต่อายุ 3 ปี ผู้ป่วยได้รับการผ่าตัดเพื่อเอานิ่วที่ไตออก 2 ครั้ง คือเมื่ออายุ 3 ปี 6 เดือน และอายุ 5 ปี ตามลำดับ ผู้ป่วยได้รับการวินิจฉัยเบื้องต้นว่าเป็นโรคซิสตินยูเรีย จากการตรวจพบผลึกลักษณะ hexagonal plate ในปัสสาวะที่ทำให้เป็นกรด และการตรวจทดสอบ urine cyanide–nitroprusside พบว่าได้ผลบวกสำหรับโรคซิสตินยูเรีย ต่อมาได้วิเคราะห์หาปริมาณ amino acids ในปัสสาวะโดยวิธี quantitative ion–exchange chromatography พบว่ามีปริมาณของ cystine และ dibasic amino acids ชนิด lysine และ ornithine เพิ่มขึ้น จึงให้การวินิจฉัยได้ว่าเป็น cystinuria ผู้ป่วยได้รับการรักษาเพื่อทำให้สาร cystine ในปัสสาวะละลายได้ดีขึ้น โดยการให้ยา potassium citrate เพื่อทำให้ปัสสาวะเป็นด่าง ร่วมกับการดื่มน้ำเป็นปริมาณมาก นิ่วในไตทั้ง 2 ข้าง ยังคงมีเพิ่มขึ้นและขนาดใหญ่ขึ้น ดังนั้นหลังการผ่าตัดครั้งที่ 2 ผู้ป่วยได้รับการรักษาเพื่อป้องกันการกลับเป็นซ้ำของนิ่ว cystine ด้วย การให้ยา D–penicillamine เพิ่มขึ้น ร่วมกับการดื่มน้ำปริมาณมากและ urine alkalinization ได้ติดตามการรักษาดังกล่าวในระยะเวลา 18 เดือนต่อมา พบว่ายังมีนิ่วที่ไตเกิดขึ้นใหม่แต่นิ่วมีขนาดเล็ก และจากการสืบค้นพบว่ารายงานนี้เป็นรายงานผู้ป่วยเด็กโรค cystinuria รายแรกในประเทศไทย

คำสำคัญ : นิ่วที่ไตชนิดเป็นซ้ำ, ซิสตินยูเรีย, ยาเพนิซิลลามีน, ปัสสาวะเป็นด่าง

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บทความที่ 13

Molecular Diagnosis of Dysmorphic Syndromes and Inherited Metabolic Disorders in Thailand

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Abstract

While dysmorphic syndromes and inherited metabolic disorders are individually rare, they collectively account for a significant proportion of illnesses, especially in children. They present clinically in a wide variety of ways, involving virtually any organ or tissue of the body making them relatively difficult to diagnose. However, reaching an accurate diagnosis for children with dysmorphic features and suspected inherited metabolic disorders is important to them and their families both for treatment and for the prevention of disease in other family members. It also makes all the accumulated knowledge available about the relevant condition.

Molecular techniques have kindled a revolution in the diagnosis of genetic disorders, including dysmorphic syndromes and inherited metabolic disorders. Molecular methods essentially avoid problems of other techniques. This review exemplifies some of the diseases that can be diagnosed by molecular tools available in Thailand and illustrates some of their benefits.

Key word : Molecular Diagnosis, Dysmorphic Syndromes, Inherited Metabolic Disorders

SHOTELERSUK V
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Dysmorphic syndromes and inherited metabolic disorders are two groups of human diseases that have long been relatively difficult to diagnose and treat, making them bitter pills for many physicians. Recent advances in molecular techniques in association with the Human Genome Project (HGP) have led to the identification of several human disease genes. One of its benefits would be for diagnosis of these

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diseases. This article exemplifies some of the diseases that can be diagnosed by molecular tools available in Thailand and illustrates some of their benefits.

Molecular diagnosis

Molecular techniques have kindled a revolution in the diagnosis of genetic disorders. In the past, genetic diagnosis was based solely on clinical features, cytogenetic methods, or biochemical tests. Clinical criteria, however, can be indistinct. In addition, some findings may develop later in life, resulting in long periods of ambiguity in the diagnosis. Cytogenetic tests can be used to diagnose only diseases with chromosomal abnormalities, which are not the majority of genetic diseases. Biochemical tests can produce ambiguous results and usually require expensive and invasive studies. Moreover, clinical criteria and biochemical tests have important limitations when used to identify carriers or make a prenatal diagnosis.

Molecular methods essentially avoid these problems. Such methods can unequivocally determine the presence or absence of a gene mutation in a patient or carrier. Because one's genetic material virtually does not change during lifetime, a molecular diagnosis can be made far in advance of the development of clinical symptoms. It requires only a sample of DNA, which is present in any nucleated cells, such as peripheral blood leukocytes. Therefore, there is no need for invasive procedures, for instance a biopsy of affected tissues. Prenatal diagnosis can possibly be made by obtaining chorionic villus or amniotic-fluid cells. Moreover, due to the high specificity of molecular diagnostic testing, screening populations for carriers is possible for some diseases.

Theoretically, molecular diagnosis of any disease whose responsible genes have been identified can be made in Thailand. But practically, due to the heterogeneity and the nature of genetic changes that underlie the disorders, molecular testing for different diseases has different levels of difficulty. Therefore, for disorders that are relatively homogeneous at the molecular level, molecular tests can be performed rapidly (within one day) and inexpensively (could cost in the same range as an echocardiogram or a skeletal survey). But for diseases whose molecular defects are extremely heterogeneous, their molecular testing can be a daunting task. Nonetheless, heterogeneity of molecular defects of majority of diseases lies between these two extremes.

Dysmorphic syndromes

Dysmorphology is the branch of clinical genetics in which clinicians study the patterns of structural defects. Although dysmorphic syndromes are individually rare, collectively they comprise a high proportion of the conditions that affect child health. Reaching an accurate diagnosis for children with dysmorphic features is important to them and their families. It makes available all the accumulated knowledge about the relevant condition.

Molecular techniques allow us to improve our ability to make precise syndrome diagnoses. Unfortunately, responsible genes for many dysmorphic syndromes have not been identified. In fact, several apparently new syndromes have continued to be described. Just early in this year, the authors described an apparent new syndrome in two Thai siblings with postnatal-onset growth deficiency, microcephaly, cataract, prominent supraorbital ridge, large joint contractures, severe osteoporosis, cortical dysplasia, cerebellar atrophy, and mental retardation[1]. The description of this new syndrome opens another way of research for the benefits of affected patients and their families.

For syndromes that have long been delineated, more phenotypic features and clinical findings continue to be added. Hydrolethalus syndrome is an autosomal recessive disorder characterized by hydrocephalus, micrognathia, limb anomalies and several other abnormalities, mostly in the midline structures. The syndrome was prevalent in Finland, where all of the Finnish patients were stillborn or died during the first day of life. The authors recently reported a Thai girl with a milder form of hydrolethalus, who survived beyond the neonatal period[2].

Diagnoses of dysmorphic syndromes whose molecular defects have not been identified still largely depend on clinical criteria. Kabuki syndrome is a good example. It was first described more than 20 years ago but its true cause remains unknown[3].

Thanks to the HGP, scientists have recently identified primary defects for several dysmorphic syndromes. One of the methods to identify the disease gene is to study monozygotic twins who are discordant for the phenotype[4]. After the disease gene is found, mutation analyses and genotype-phenotype correlations can be studied. Cystinosis is an autosomal recessive lysosomal storage disorder characterized by renal Fanconi syndrome and corneal cystine crystal

deposition. There are three types of the disease classified mainly on the age of onset and severity of the disease. After the disease gene was identified[5], all of the three types were found to be allelic[6,7] with some correlations between the positions of the genetic changes and the phenotypic features[8-10]. Hermansky-Pudlak syndrome is an autosomal recessive disease with albinism and bleeding diathesis[11]. Mutations in at least four genes can cause the disease[12,13]. Phenotypic features from each gene are somewhat different[14-16]; for example, patients with mutations in *HPS2* gene tend to have neutropenia, which does not usually occur in patients with mutations in other genes[17].

Thai patients with many syndromes have had similar clinical and molecular features to other populations. These include multiple endocrine neoplasia type 2A[18], achondroplasia[19], Crouzon syndrome[20], Apert syndrome[20] and Pfeiffer syndrome [21] (Fig. 1). These data can be used in several ways including prenatal diagnosis[22].

Some Thai patients, however, have distinct clinical features even with exactly the same mutations as other patients of different ethnic groups. The authors studied a 15-year-old Thai boy with an unspecified craniosynostosis syndrome who was found to be heterozygous for a 870G->T change in the *FGFR2* gene. This mutation has previously been reported in a Caucasian patient with severe Pfeiffer syndrome type 2 that is distinct from the craniosynostosis in the Thai patient[23].

Several Thai patients have been found to have unique genetic changes. These include Van der Woude syndrome, the most common autosomal dominant cleft syndrome characterized by cleft lip and palate with lip pits[24] and pseudoachondroplasia, an autosomal dominant skeletal dysplasia with precocious osteoarthritis[25]. In addition, the authors found a Thai patient with nasopharyngeal carcinoma (NPC) with a mutation in *FGFR3* gene. It was the first time an *FGFR3*'s role was demonstrated in the development of human NPC[26].

Towards the completion of the HGP and the availability of single nucleotide polymorphisms in various ethnic groups, not only genetic defects of single gene disorders can be studied, but also attempts to understand further the genetic components of multifactorial disorders are more feasible and fruitful. The authors recently found that a maternal 677CT/1298AC genotype, a polymorphism in the *MTHFR* gene, is a risk factor for having children with cleft lips with the odds ratios of 4.43 (95% confidence interval, 1.33-15.10). Approximately 12 per cent of Thai mothers whose children had cleft lips had such genotype. Therefore, folate supplement in a pregnant woman's diet may benefit 12 per cent of Thai children who are susceptible to CL/P due to the 677CT/1298AC genotype in their mothers[27].

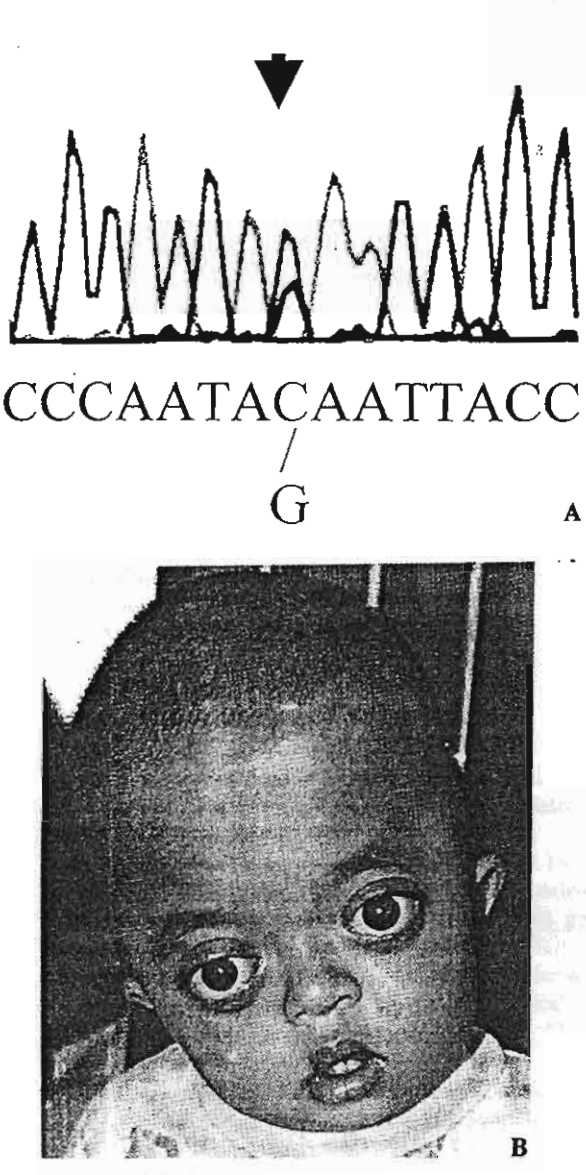

Fig. 1. **The antisense sequence electropherogram (A) showing a heterozygous *FGFR2* S347C (1040C>G) mutation (arrow) helps confirming the diagnosis of Crouzon syndrome in a Thai girl with brachycephaly, exorbitism and maxillary hypoplasia (B).**

Inherited metabolic disorders

Inherited metabolic disorders or inborn errors of metabolism are a group of disorders with defects in catabolic or anabolic pathways of nutrients. While they are individually rare, they collectively account for a significant proportion of illness, especially in children. They present clinically in a wide variety of ways, involving virtually any organ or tissue of the body. Accurate diagnosis is important both for treatment and prevention of disease in other family members.

Due to its unique defective point in the metabolic pathways, diagnosis of each of the diseases usually requires distinctive methods. A few disorders can be diagnosed with imaging studies; for example, Pompe disease, an autosomal recessive disorder with a defect in acid α-glucosidase leading to an accumulation of glycogen in lysosomes. Such accumulation gives a diagnostic feature under electron microscopy (28). In addition, a prenatal diagnosis for this deadly disorder can be made by this method using cells from chorionic villus sampling or amniocentesis(29).

Disorders of small molecules, such as aminoacidemia, aminoaciduria, and organic acidemia can usually be provisionally diagnosed by analyzing plasma amino acid, urine amino acid(30) or urine organic acid profiles(31). For storage disorders and others including methemoglobinemia, essays to determine enzymatic activities are the standard diagnostic methods(32). In Thailand, however, laboratories offering such methods are scarce. On the contrary, laboratories that are able to perform molecular studies for diagnosis of these diseases are more available(33). Actually, the methods have been used to confirm diagnosis of many disorders in developed countries(34). Therefore, molecular methods should be considered as an alternative means to diagnose patients with inherited metabolic disorders in Thailand.

Molecular techniques will become more and more important diagnostic tools for assisting Thai physicians to diagnose and manage patients with dysmorphic syndromes and inherited metabolic disorders.

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การวินิจฉัยกลุ่มอาการที่มีความผิดปกติทางรูปร่างและกลุ่มโรคพันธุกรรมเมแทบอลิก ด้วยวิธีทางอณูพันธุศาสตร์

*วรศักดิ์ โชติเลอศักดิ์, พบ**

โรคในกลุ่มอาการที่มีความผิดปกติทางรูปร่างและกลุ่มโรคพันธุกรรมเมแทบอลิก แม้แต่ละโรคจะพบได้น้อย แต่โดยรวมแล้วนับเป็นสัดส่วนที่สำคัญของการเจ็บป่วย โดยเฉพาะอย่างยิ่งในเด็ก โรคกลุ่มนี้แสดงอาการได้ในทุกอวัยวะ ทุกรูปแบบ ทำให้การวินิจฉัยทำได้ค่อนข้างยาก อย่างไรก็ตามแพทย์จำเป็นต้องพยายามให้การวินิจฉัยที่ถูกต้อง ทั้งนี้เพื่อประโยชน์ในการรักษาผู้ป่วยและป้องกันการเกิดซ้ำของสมาชิกคนอื่นในครอบครัว

วิธีทางอณูพันธุศาสตร์ได้ปฏิวัติวิธีการวินิจฉัยโรคพันธุกรรมรวมทั้งโรคในสองกลุ่มนี้ วิธีดังกล่าวสามารถหลีกเลี่ยงจุดด้อยของวิธีการวินิจฉัยอื่น ๆ บทความนี้ได้ยกตัวอย่างโรคบางโรคในกลุ่มนี้ที่สามารถให้การวินิจฉัยได้ด้วยวิธีทางอณูพันธุศาสตร์ที่ทำได้แล้วในประเทศไทย และชี้ให้เห็นถึงประโยชน์ที่ได้ตามมาบางประการ

คำสำคัญ : การวินิจฉัยด้วยวิธีทางอณูพันธุศาสตร์, กลุ่มอาการที่มีความผิดปกติทางรูปร่าง, กลุ่มโรคพันธุกรรมเมแทบอลิก

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บทความที่ 14

A novel mutation of the *COMP* gene in a Thai family with pseudoachondroplasia

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Abstract. Pseudoachondroplasia (PSACH) is an autosomal dominant disorder characterized by disproportionate short stature and precocious osteoarthritis. Radiographic manifestations include epiphyseal, metaphyseal and vertebral abnormalities. Mutations in the cartilage oligomeric matrix protein (*COMP*) have been identified to cause PSACH. Most of them affect one of the eight calcium-binding domains of *COMP*. We describe a clinically and radiologically typical PSACH 4-year-old girl and her 31-year-old father. A novel mutation, 1345-1347CCC deletion in exon 13, of *COMP* was identified in both patients. The deletion would be expected to result in the loss of the conserved proline at codon 449 from the sixth calcium-binding domain. This result further supports that *COMP* is the only gene, discovered to date, responsible for PSACH across different populations and that the calcium-binding domains are important to the function of the normal *COMP*.

Introduction

Pseudoachondroplasia (PSACH) is an autosomal dominant disorder characterized by disproportionate short stature, excessive ligamentous laxity, and precocious osteoarthritis with normal face and intelligence (OMIM 177170). Radiographic manifestations include epiphyseal, metaphyseal and vertebral abnormalities (1,2). Tubular bones are short, metaphyses are irregular and widened, epiphyses are small and fragmented, and vertebrae demonstrate flattening and anterior beaking. In 1995, it was demonstrated that mutations in the cartilage oligomeric matrix protein (*COMP*) cause PSACH (3,4). At least 48 mutations have been identified, almost all affecting one of the eight calcium-binding domains. We describe a PSACH Thai girl and her father with a novel mutation in a calcium-binding domain, emphasizing its importance to the normal function of *COMP*.

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Key words: pseudoachondroplasia, cartilage oligomeric matrix protein, mutation analysis

Materials and methods

Case report. A female infant was born at term by cesarean section due to cephalopelvic disproportion to a 27-year-old G1P0 Thai mother and a 27-year-old unrelated Thai father. The pregnancy was uncomplicated. Birth weight was 3,000 g and birth length was 50 cm. APGAR scores at 1 and 5 min were 10 and 10, respectively. Physical examination at birth was unremarkable. She had been healthy until she was 18 months old when her parents first noticed that she had short stature, bowed legs and waddling gait. At age 3 years, she could ride a tricycle, speak in sentence, and tell a story. Her father was short but her mother had normal stature. The father completed his bachelor degree in computer. He had complaint of occasional back and knee pains. However, he had never undergone surgery. Besides the 2 individuals, there were no other family members with short stature. History of consanguinity was denied.

Physical examination of the girl at age 4 years revealed height of 83.8 cm (-5 SD), weight 12.3 kg (-2 SD) and OFC 49.5 cm (mean). Arm span was 76.3 cm. The upper to lower trunk ratio was 1.51. Her craniofacial appearance was normal. The lengths of her arms were 12.5 cm, forearms 10 cm, total hands 10 cm and palms 6 cm. The extensions of her shoulder, elbow, hip and knee joints were limited. Flaring and ulnar deviations of her wrists were present. She had genu varum and mild scoliosis (Fig. 1A and B).

Physical examination of her father at age 31 years revealed height of 113 cm, OFC 58 cm, arm span 102 cm, upper to lower trunk ratio 2.05, arms 20.5 cm, forearms 14.5 cm, total hands 12.8 cm, and palms 7.5 cm. His craniofacial appearance was normal. He had mild pectus excavatum and mild scoliosis. The extensions of his shoulder, elbow, hip and knee joints were limited (Fig. 1C and D).

Radiographic findings of the patient revealed short tubular bones, irregular and widened metaphyses, small and fragmented epiphyses, varus deformity of elbows and knees, bullet-shaped vertebral bodies, small odontoid process and slant and irregular acetabuli (Fig. 2A, C, and E). Radiographic manifestations of her father were similar, but much more severe, to his affected daughter (Fig. 2B, D, and F).

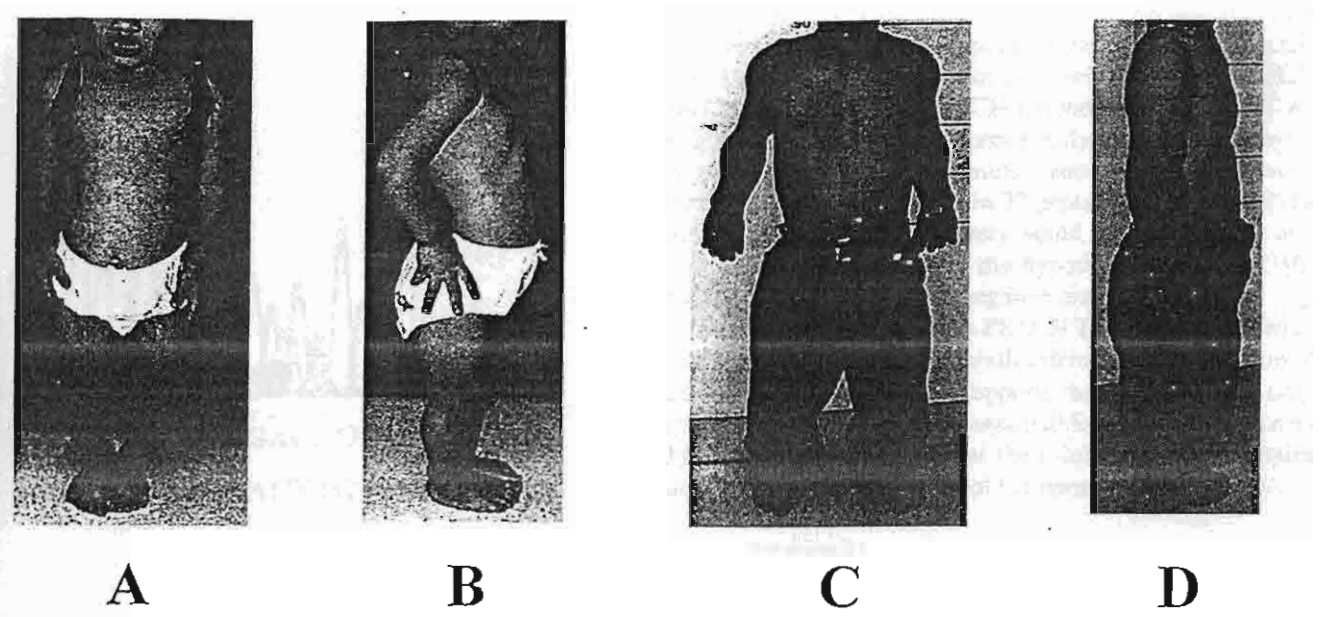

Figure 1. The patient (A and B) and her father (C and D). Total body (A and C): note the normal facial appearance, short limbs, and genu varus. Side (B and D): note the lumbar lordosis and the slight ulnar deviations of wrists.

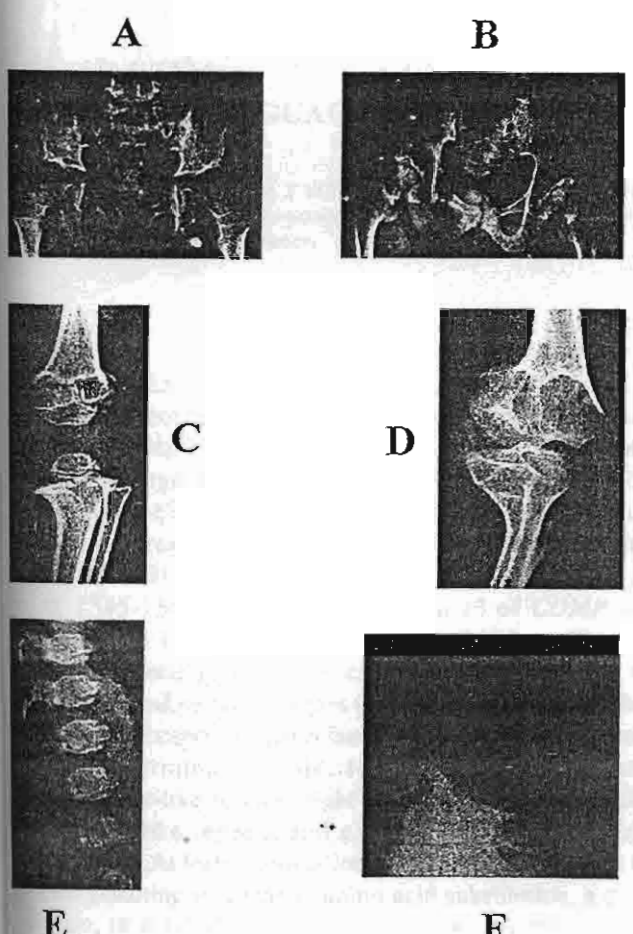

Figure 2. Radiographs of the patient (A, C, and E) and those of her father (B, D, and F). Hips (A and B): note that the acetabuli are irregular, the epiphyses (A) and the femeral heads (B) are small. Left knee (C and D): note the abnormal epiphysis and metaphysis in the child (C). Spine (E and F): note the flattened, bullet-shaped vertebrae (E) and the anterior wedging of L2 and the severe lordosis of the lumbosacral junction (F).

Results

After informed consent was obtained in accordance with the standards set by local institutional review boards, 3 ml of peripheral blood of the patient and her parents were obtained for DNA isolation by a standard method. Because most identifiable mutations in patients with pseudoachondroplasia are in exon 13 (exon 17B of the thrombospondins) (5) of *COMP*, we designed a nested PCR with the forward primer: 5'-TGGAGAGCTCATTGTCTCTG-3' and the backward primer: 5'-ACCTTGTCTGCATCAAAGTCG-3' for the first round PCR, giving a 385 bp product. For the second round PCR, we used the forward primer: 5'-TCCCACCTATCC ACTCT-3' and the backward primer: 5'-GCCCGCCCACCG TAGAC-3' to amplify a 276 bp product. For both round PCR amplifications, 35 cycles were performed at 94°C for 30 sec, 58°C for 30 sec, and 72°C for 45 sec, followed by an additional extension step at 72°C for 10 min. The PCR products were electrophoresed on a 2% agarose gel (Promega) and stained with ethidium bromide. The visualized bands were extracted and purified with a kit (Bio 101), and sequenced in both directions by using an automated DNA sequencer (ABI Prism 310 Genetic Analyser, Perkin Elmer).

Direct sequencing analysis of the PCR products revealed that both patients were heterozygous for a deletion of 3 bp, CCC, from nucleotide 1345 to 1347 (Fig. 3). Sequence of the girl's mother was normal.

Discussion

We describe a clinically and radiologically typical PSACH 4-year-old girl and her 31-year-old father. The girl's birth weight and length were within the normal ranges. The initial features were a short stature and an abnormal gait, appearing in her toddler stage. The father had premature severe osteo-

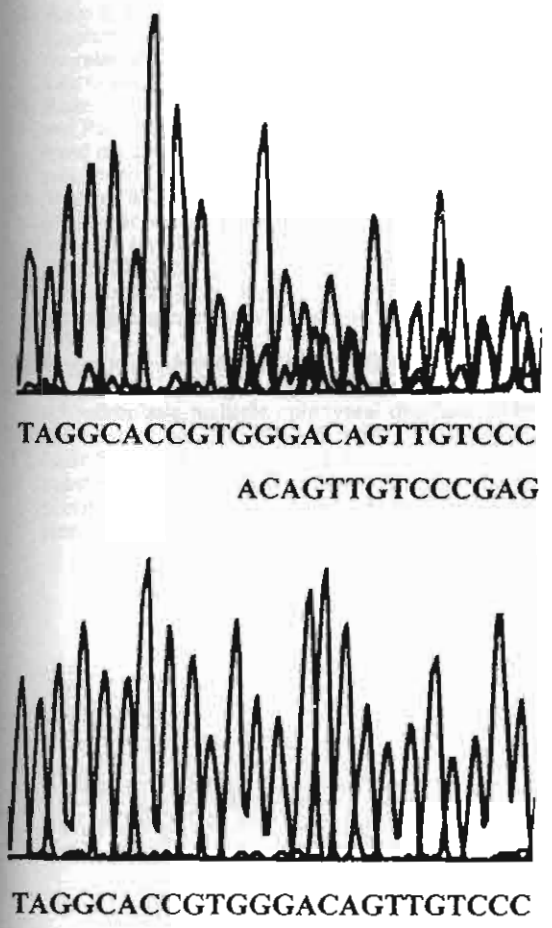

Figure 3. Sequencing from the 3' to 5' direction of the patient (A) and her mother as a control (B). Note the normal sequence can be observed along with the mutant (3 bp deletion) sequence.

arthritis but had not undergone any operations and had no extraskeletal complications, consistent with the typical natural history of patients with PSACH (6). Our patients demonstrated bowing of legs, the most common skeletal complication occurring in 83.8% of patients with PSACH (6). Odontoid hypoplasia present in both patients occurs in approximately half of PSACH individuals (7).

A 1345-1347CCC deletion in exon 13 of *COMP* was identified in both patients. Human *COMP* is a 524-kDa homopentameric glycoprotein expressed prominently in the matrix surrounding chondrocytes (8,9). It is one of the members of the thrombospondin gene family. The monomer contains an amino-terminal domain, four contiguous epidermal growth factor-like repeats, eight contiguous calcium-binding calmodulin-like repeats, and a carboxyl-terminal globular domain (10). At least 48 mutations have been identified in the *COMP* resulting in a single amino acid substitution, a small deletion, or a small insertion. Most known mutations affect residues in the calcium-binding domains with only 4 mutations locating in the carboxyl-terminal domain (3,4,11-22). The 3-bp deletion found in our patients would be expected to result in the loss of the conserved proline at codon 449 from the sixth calcium-binding domain. This proline residue is present in five of the eight calcium-binding domains. A mutation at the same codon, P449T resulting from a 1345C→A mutation, was previously reported to be responsible for PSACH in a family (19). However, no mutations in the other four proline residues have been identified in PSACH individuals. The loss of the residue in our patients would presumably affect the structure of the polypeptide chain. If the mutant and the normal monomer are equally produced, only 1 in 32 pentameric COMP will be completely normal, while others would have at least one arm with a mutation, supporting the hypothesis that the *COMP* mutations act in a dominant negative manner (3,4).

In summary, we describe a PSACH Thai girl and her father with a novel mutation in the sixth calcium-binding domain of *COMP*. This result further supports that *COMP* is the only gene, discovered to date, responsible for PSACH across different populations and that the calcium-binding domains are important to the function of the normal *COMP*.

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Brief communication

Novel mutations in a Thai patient with methylmalonic acidemia

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Abstract

A Thai patient with methylmalonic acidemia (MMA) and no methylmalonyl-CoA mutase (MCM. EC 5.4.99.2) activity in leukocytes in the presence of deoxyadenosyl cobalamin (mut^0) was found to be heterozygous for two novel mutations: 1048delT and 1706_1707delGGinsTA (G544X), inherited from her mother and father, respectively. The proband was also heterozygous for the polymorphism, A499T, which did not affect the activity of recombinant MCM.

Introduction

Methylmalonic acidemia (MMA, MIM 251000) is a form of metabolic acidosis caused by a defect in propionate metabolism at the step of conversion of methylmalonyl-CoA to succinyl-CoA [1]. MMA is caused by a functional defect in the methylmalonyl-CoA mutase (MCM, EC 5.4.99.2), which converts L-methylmalonyl-CoA to succinyl-CoA, due either to a mutation of its gene (mut^0 or *mut*) or to a defect in metabolism of its cofactor, deoxyadenosyl cobalamin (*cbl A-H*) [1–3].

So far, over 50 disease-causing *mut* mutations have been identified, along with many polymorphisms [4–10]. Several *mut* mutations have been identified in Japanese patients [11,12], but relatively little has been done in the rest of Asia. Here, we have identified the first Thai case of mut^0 MMA to be confirmed at the molecular level, identifying two novel mutations.

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Patient and methods

Patient

The female infant of unrelated Thai parents presented at age 2 days with tachypnea and lethargy. Laboratory data indicated severe metabolic acidosis with a very wide anion gap. Urine organic acid analysis by GC-MS [13] revealed marked elevation of methylmalonic acid.

Enzyme assay

Leukocytes were extracted and MCM activity assayed as previously described with reduced reagent volumes [14,15]. The K_m of methylmalonyl-CoA was determined in 105 μM deoxyadenosyl cobalamin, while the K_m of deoxyadenosyl cobalamin was determined in 380 μM methylmalonyl-CoA.

MCM gene amplification and sequencing

Total RNA was extracted from the leukocytes using a Qiagen blood RNA kit (Qiagen GmbH, Hilden,

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Germany). The MCM cDNAs were amplified by RT-PCR, as previously described [4], and directly sequenced. For analysis of the allelic segregation, the cDNA were cloned into pGEMT vectors (Promega, Madison, WI) and sequenced. Genomic DNA was prepared using a Qiamp DNA minikit (Qiagen). To analyze the 1048delT mutation, exon 5 was PCR amplified using the For987 and Rev1131 primers [4]. Exons 8 and 9 were amplified with the flanking primers, Ex8F (5′-GAAAATACATC ATAACCAGAGCA-3′) and Ex8R (5′-TAATACACA CCTCATGCTGTTG-3′) for exon 8, and Ex9F (5′-CA TCAGGGTCTAATCTCTTGAT-3′) and Ex9R (5′-TC ACATGGTTTACAGGATCAAC-3′) for exon 9, to detect the A499T and G544X mutations, respectively. The 1048delT mutation was confirmed by cleavage of the exon 5 PCR product with *Alu*I restriction endonuclease (New England BioLabs, Beverly, MA).

MCM expression in Escherichia coli

The mRNA of the proband and a normal control were reverse-transcribed and PCR amplified using *Pfu* polymerase (Promega) and the primers MCMF-*Nco*I (5′-AT TTCCATGGTACACCAGCAACAGCCCCT-3′) and MCMR-*Sac*I (5′-ATTTGAGCTCTCTCTTCTTTGAT CATAACTA-3′) to add *Nco*I and *Sac*I sites, cloned into these sites in pET32a and pET23d (Novagen, Madison, WI), and sequenced. To isolate the A499T and G544X mutations from other mutations and PCR errors, nucleotides 1160–1741 containing these mutations were excised with *Bam*HI and *Nsi*I, and ligated into the corresponding sites in the MCM cDNA expression vector to create single mutant expression vectors. These constructs were used for protein expression, and the *E. coli* cell extracts assayed for MCM activity and protein content, as previously described [16].

Results and discussion

The proband had typical clinical presentation and urine organic acid pattern of MMA. No MCM activity could be detected in leukocyte extracts from the proband, whereas activity was detected in all normal controls (121 ± 50 pmol succinyl-CoA produced/min/mg protein) and in the parents (78 and 52 pmol/min/mg for the mother and father, respectively).

The proband's cDNA had three heterozygous nucleotide changes: 1571G > A (A499T), 1706G > T, and 1707G > A, with the later two on the same allele to give 1706_1707delGGinsTA (G544X), and one heterozygous single base deletion, 1048delT. The previously described polymorphism H532R [5] was homozygous in all cDNA from this family and in three Thai controls. None of the new mutations were clearly detected in the mRNA from the parents, but genomic DNA sequence showed that the mother was heterozygous for the 1048delT and A499T mutations, while the father was heterozygous for the G544X mutation. The presence of the 1048delT mutation in the proband and her mother, but not the father, could be confirmed by PCR amplification of exon 5, followed by *Alu*I digest. The mutation eliminates an *Alu*I site, resulting in only approximately half the PCR product being digested in the mother and patient. The inability to detect the mutations in the parents' mRNA may indicate that the mutant mRNAs are less stable than the normal MCM mRNA. The 1048delT deletion causes a frameshift at Ala324, resulting in a change of the next eight residues from GRRLWAHL to VEDSGLT (stop), so both new mutations result in premature stop codons. The instability of MCM mRNA with premature stop codons has been noted in the past for other mutations resulting in premature stop codons [11].

The A499T change in this patient would not have any effect, since it comes after the 1048delT frameshift, but it is unclear whether it might affect other patients. Berger et al. [17] reported it in association with the mutation IVS8 + 3a > g, which apparently caused a high frequency of incorrect splicing. They suggested that the A499T mutation had no affect, since the position is not evolutionarily conserved. This mutation did not seem to affect the splicing, since no mis-spliced mRNA was detected here. MCM specific activities in extracts of *E. coli* expressing thioredoxin–MCM fusion proteins with normal MCM cDNA and A499T cDNA were high and similar (8.53×10^3 and 8.11×10^3 pmol succinyl-CoA/mg/min), while those with the G544X mutation had no activity. Expression of MCM without the N-terminal thioredoxin fusion protein gave similar results. The K_m values of the normal and A499T MCM for the cofactor, deoxyadenosyl-cobalamin, in the presence of 0.38 mM substrate were 0.26 and 0.19 μM, respectively, while K_m values for the substrate, methylmalonyl-CoA, were 0.13 and 0.14 mM, respectively. Thus, the A499T MCM enzyme appeared normal in terms of binding cofactor and substrate and catalyzing the mutase reaction. Analysis of 100 Thai controls found this polymorphism represented 8.0% of the alleles (16 of 200 chromosomes).

The patient appeared to be a compound heterozygote for two new mutations, 1048delT and G544X. Both mutants are expected to produce a protein with a truncated MCM domain and no cobalamin-binding domain [18,19], so no MCM activity is expected. The A499T polymorphism, however, seemed to produce a normal enzyme in the recombinant system, and was found to be a frequent allele in the normal Thai population.

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บทความที่ 16

A novel mutation, 1234del(C), of the *IRF6* in a Thai family with Van der Woude syndrome

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Abstract. Van der Woude syndrome (VWS) is an autosomal dominant disorder and the most common cleft syndrome characterized by cleft lip and palate with lip pits. Very recently, mutations in the interferon regulatory factor 6 gene (*IRF6*) were identified to cause VWS in patients of northern European descent. We describe a Thai family with VWS. The proband, an 8-month-old boy, had bilateral complete cleft lip and palate, and two conical elevations with lip pits on his lower lip. Four other family members had various manifestations of the clefts and lower lip pits. Mutation analysis of the proband and his mother for the entire coding region of *IRF6* identified a novel mutation, 1234del(C), in its exon 9. The deletion is expected to result in some amino acid changes followed by truncation at amino acid 435. This observation supports that *IRF6* is the gene responsible for VWS across different populations and that haploinsufficiency of the gene disturbs development of the lip and palate.

Introduction

Van der Woude syndrome (VWS, OMIM 119300) is an autosomal dominant disorder characterized by cleft lip and palate with lip pits. It is the most common syndromic form of oral clefts (1). Most reported familial VWS cases have been linked to 1q32-q41 (2), but a second locus has been mapped to 1p34 (3). Very recently, mutations in the interferon regulatory factor 6 gene (*IRF6*) were demonstrated to cause VWS. So far, forty-six mutations in *IRF6* associated with VWS have been identified (4). Here we describe a Thai family with VWS with a novel mutation in exon 9 of *IRF6*.

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Key words: Van der Woude syndrome, interferon regulatory factor 6, mutation analysis

Materials and methods

Case report. The proband, a boy, was born after uncomplicated pregnancy at term by spontaneous vaginal delivery to a 38-year-old G2P1 Thai mother and a 30-year-old unrelated Thai father. Birth weight was 2,500 g. He was noted to have oral clefts since birth. His development was appropriate for age. Physical examination at the age of 8 months revealed length of 64 cm (-2 SD) and weight 7 kg (-1.5 SD). He had bilateral complete cleft lip and palate, and two conical elevations with lip pits on his lower lip (Fig. 1A).

His mother had cleft lip on the right side, bilateral complete cleft palate and lip pits on her lower lip (Fig. 1B). Three other members in the family, i.e., the maternal grandfather, an uncle and his daughter (first cousin) had various manifestations of the clefts and lower lip pits (Fig. 2).

Mutation analysis. After informed consent was received, peripheral blood (3 ml) was obtained from the boy and his mother and DNA was extracted by standard methods. Exons 3 to 8 and a part of exon 9 of *IRF6*, which contain its entire coding region, were amplified by standard PCR (4). The PCR products were treated with ExoSAP-IT (USP Corporation, Cleveland, Ohio), according to the company recommendations, and sent for direct sequencing at the National Science and Technology Development Agency, Bangkok, Thailand.

Results and Discussion

Direct sequencing analysis of the PCR products revealed that the boy and his mother were heterozygous for deletion of cytosine at nucleotide position 1,234 [1234del(C)] in exon 9 of *IRF6* (Fig. 3). The mutation is expected to result in subsequent changes of 24 amino acids and truncation at amino acid 435 because of a frame shift.

In our family, the proband and his mother had clinically typical VWS. Both had cleft lip, cleft palate and lip pits, similar to the boy's affected first cousin (Fig. 2). However, the proband's maternal grandfather had cleft lip and lip pits, while a proband's uncle had only lip pits. This variable expressivity has been known in this syndrome. Previous studies showed that lip pits were the most common manifestation, being present

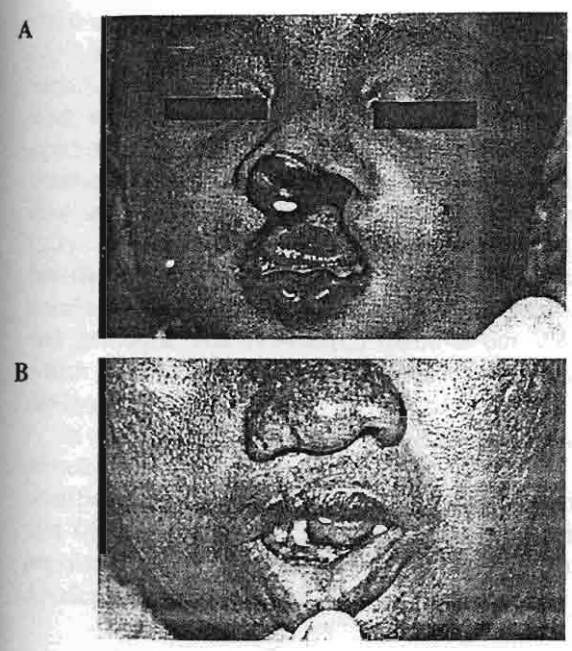

Figure 1. A, the proband has cleft lip, cleft palate and lip pits. B, the proband's mother has surgically repaired cleft lip, unrepaired cleft palate and a lip pit on her lower lip.

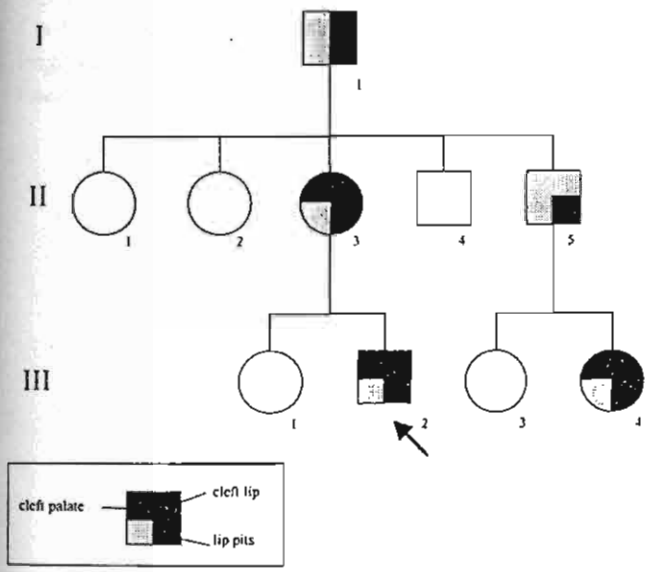

Figure 2. Pedigree of the family. Unaffected individuals (open symbol), proband (arrow), and individuals with VSW (gray) are indicated. Symbols representing specific manifestations are shown below the pedigree.

in 88% of affected individuals and the only manifestation in 64% of them (5). Oral clefts occurred in 21% and penetrance was 96.7% (5). All five affected members in our family were in one clefting phenotype, i.e., the cleft lip with or without cleft palate (CL/P). The CL/P and isolated cleft palate (CP) are genetically distinct. The mixed phenotype with CL/P and CP in the same family is very rare in non-syndromic oral clefts and not found in the majority of patients with syndromic clefts (6). Although not seen in our family, VWS is one of

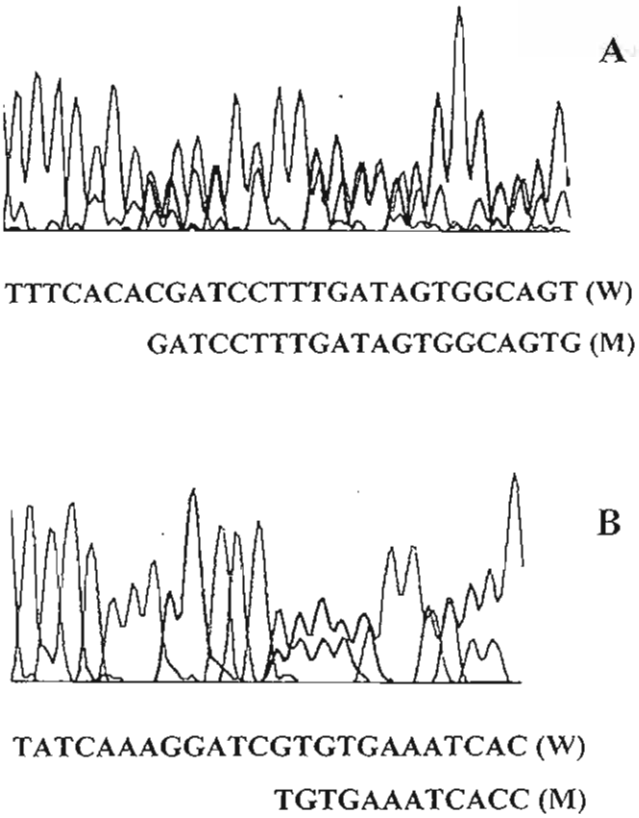

Figure 3. A, the sense and B, the antisense sequence electropherograms in the proband. Note the wild-type sequence (W) can be observed along with the mutant sequence (M).

such few syndromic forms of oral clefts that manifest either clefting phenotype.

The IRF6 protein is one of the nine members in a family of transcription factors. They share a highly winged-helix conserved DNA-binding domain (amino acids 13-113) and a less conserved protein-binding domain (amino acids 226-394) named the Smad-interferon regulatory factor-binding domain (SMIR). No obvious functional domain has been found in the C-terminus region. However, since the mutation identified in our family was a heterozygous 1234del(C) that results in a truncated protein without 56 amino acids at the C-terminus, certain functional domain(s) may exist in this region. In fact, Kondo *et al* (4) also reported a missense mutation in the C-terminus region.

IRF6 presumably forms dimers before it binds to other transcription factors and their DNA targets (7). Most IRF are regulators of host defense after viral infection (8). Distinctively, IRF6 functions in a pathway for normal development of the lip, palate, skin and external genitalia (4). IRF binding sites were found in the promoter of *MSX1*, mutations of which are associated with orofacial clefting (9). In addition, IRF6 may interact with Smads, a family of transcriptional factors known to transduce TGF-ßs, the gene product of which is required for palatal fusion (10). These observations suggest that IRF6, MSX1, and TGF-ßs may be involved in a common pathway.

Previous studies demonstrated that protein-truncation mutations, as observed in our family with VWS, and large

deletions encompassing the entire *IRF6* resulted in VWS (4,11,12). On the other hand, missense mutations in the DNA-binding domain that directly contact DNA were associated with another dysmorphic syndrome, popliteal pterygium syndrome (PPS; OMIM 119500). PPS is another autosomal dominant disorder characterized by orofacial manifestations similar to VWS as well as by skin and genital abnormalities (13). Therefore, it has been hypothesized that haploinsufficiency of *IRF6* disrupts orofacial development, while dominant-negative mutations disturb development of the skin and genitalia. The 1234del(C) found in our VWS family, which is expected to result in a truncation protein, supports the hypothesis.

In summary, we describe a VWS Thai family with a novel protein truncation mutation presumably resulting in haploinsufficiency of the *IRF6*. This observation further supports that *IRF6* is the gene responsible for VWS across different populations and that haploinsufficiency of the gene disturbs development of the lip and palate.

Acknowledgments

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บทความที่ 17

Scientific Foundations

FGFR2 Mutations among Thai Children with Crouzon and Apert Syndromes

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Crouzon and Apert syndromes have been reported to be associated with mutations in *Fibroblast Growth Factor Receptor 2 (FGFR2)* gene in various ethnic groups, but never in Southeast Asian subjects. Therefore, the authors conducted a study to characterize 11 Thai patients: four with Crouzon syndrome and seven with Apert syndrome. All cases are sporadic. Mean paternal and maternal ages were 38.7 and 28.6 years, respectively. Molecularly, all patients were found to have mutations in the *FGFR2* gene. Three mutations (C278F, S347C, S351C) were detected in all Crouzon patients with two having S351C. The seven patients with Apert syndrome have either S252W or P253R mutation. The authors' findings that sporadic cases were associated with advanced paternal age and that they all had mutations in *FGFR2* are consistent with previous reports. This is another observation supporting the causative role of *FGFR2* mutations in Crouzon and Apert syndromes.

Key Words: Crouzon syndrome, Apert syndrome, fibroblast growth factor receptor, mutation analysis.

Crouzon syndrome (MIM 123500), one of the most common syndromic craniosynostoses, is characterized by abnormal head shape due to premature closure of the cranial sutures, prominent eyes secondary to shallow orbits, and maxillary hypoplasia.[1] Apert syndrome (MIM 101200), one of the most serious syndromic craniosynostoses, is characterized by symmetrical syndactyly of the hands and feet and abnormal craniofacial features resembling those of Crouzon syndrome.[2] Intelligence varies from normalcy to mental retardation.[3]

Both syndromes are known to be inherited in an autosomal dominant manner. Recently, an exclusive paternal origin of *de novo* mutations associated with advanced paternal age has been described.[4,5] In 1994, Crouzon syndrome was found to be associated with mutations in *Fibroblast growth factor receptor 2 (FGFR2).*[6,7] One year later, a similar association between Apert syndrome and *FGFR2* was evidenced.[8] Fibroblast growth factors (FGF) constitute a family of related mitogens. Four FGFRs (FGFR1–4) make up a family of structurally related receptors encoded by four different genes.[9] These receptors are composed of three extracellular immunoglobulin (Ig)-like domains, a transmembrane domain, and a tyrosine kinase domain.[10] The *FGFR2* gene on chromosome 10q25.3–26 consists of 20 exons spanning a region of greater than 120 kb.[11] Besides Crouzon and Apert syndromes, specific point mutations in *FGFR2* have been associated with several other craniosynostoses including Pfeiffer syndrome (MIM 101600),[12] Beare-Stevenson cutis gyrata syndrome (MIM 123790),[13] Jackson-Weiss syndrome (MIM 123150),[6] and some cases of Antley-Bixler syndrome (MIM 207410).[14]

To our knowledge, mutations in the *FGFR2* gene have been associated with both Crouzon and Apert syndromes in various ethnic groups including Japanese[15] and Taiwanese subjects,[16] but not in Southeast Asians subjects. We therefore conducted a study to clinically and molecularly characterize 11 Thai patients with craniofacial dysostosis.

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Materials and Methods

Patients were ascertained through the Genetics Clinic and Craniofacial Clinic of the King Chulalongkorn Memorial Hospital, Bangkok, Thailand during a 2-year period, January 2000 to December 2001.

The study was approved by the local ethics committee. After informed consent was obtained, 6 milliliters of peripheral blood were obtained for DNA isolation by a standard method. For patients with Crouzon syndrome, *FGFR2* exon 8 and *FGFR2* exon 10 were polymerase-chain-reaction (PCR) amplified and subsequently sequenced using previously described methods.[17] For patients with Apert syndrome, only the *FGFR2* exon 8 was amplified. Subsequently, the PCR-amplified 322-bp fragment was divided into two tubes and digested with *Mbo*I or *Bgl*I (New England Biolabs, Beverly, MA) for identification of the presence of S252W or P253R, respectively. After overnight incubation at 37°C, digested products were electrophoresed through a 12% polyacrylamide gel or 3% agarose gel before being stained with ethidium bromide. To confirm the presence of the mutations, PCR products from two patients, each with S252W or P253R, were also directly sequenced using an automated DNA sequencer (ABI Prism 310 Genetic Analyzer, Perkin Elmer, Wellesley, MA).

Results

There were 11 patients included in the study: four with Crouzon syndrome and seven with Apert syndrome. Male-to-female ratio was 3:8. Ages ranged from 2 months to 10 years. The clinical findings are summarized in Table 1. All cases were sporadic. Mean paternal and maternal ages were 38.7 and 28.6 years, respectively. Three of the patients' fathers were more than 35 years old (57, 59 and 62 for patients 5, 8 and 7, respectively) when the patients were born.

With the methods used, mutations in *FGFR2* were identified in all cases. There were three different mutations in four patients with Crouzon syndrome: one (C278F) in exon 8, and the other two (S347C and S351C) in exon 10. All seven patients with Apert syndrome had either S252W or P253R mutation (Table 1 and Figure 1).

Discussion

We identified 11 Thai children with syndromic craniosynostoses. All were sporadic. About one quarter of the reported cases of Crouzon syndrome had no family history while the majority of Apert cases were sporadic.[18] Sporadic cases presumably represented fresh mutations. The advanced paternal age of our patients supports the findings from previous reports that *de novo* mutations in both syndromes were exclusively paternal in origin and associated with advanced paternal age.[4,5]

All of the mutations identified in our patients were missense and previously described in other ethnic background studies.[19] Two patients with Crouzon syndrome (patients 3 and 4) with the same S351C mutation had severe clinical manifestations and died in their infancy. The phenotypes of previously reported cases with this S351C mutation varied from Crouzon, Pfeiffer, Antley-Bixler, or unclassified

Table 1. Clinical Features of the 11 Thai Children with Crouzon or Apert Syndrome

Patient ID	*Clinical diagnosis*	*Sex*	*Age**	*Paternal/Maternal age‡ (y)*	*Development and clinical course*	*Mutation*
1	Crouzon	F	10 y	27/22	IQ = 53, moderate mixed hearing loss	C278F (833G > T)
2	Crouzon	F	18 mo	32/24	Normal development. Sudden unexplained death at age 2 years.	S347C (1040C > G)
3	Crouzon	M	2 mo	34/33	Died of pneumonia at age 2 months	S351C (1052C > G)
4	Crouzon	F	1 y	33/34	Hydrocephalus, compressive optic neuropathy, hearing loss, died of aspiration pneumonia at age 1 year.	S351C (1052C >G)
5	Apert	M	18 mo	57/34	DQ = 67, hearing loss, cleft soft palate	P253R (758C > G)
6	Apert	F	30 mo	25/23	DQ = 46, cleft palate	S252W (755C > G)
7	Apert	F	3 y	62/37	IQ = 60	S252W (755C > G)
8	Apert	F	2 y	59/24	Incomplete cleft palate	P253R (758C > G)
9	Apert	F	3 mo	30/25	—	S252W (755C > G)
10	Apert	F	9 y	33/30	IQ = 55, cleft soft palate	S252W (755C > G)
11	Apert	M	20 mo	34/29	DQ = 89	P253R (758C > G)

*Age at last visit.
‡Parental ages when child was born.
DQ, developmental quotient; IQ, intelligence quotient.

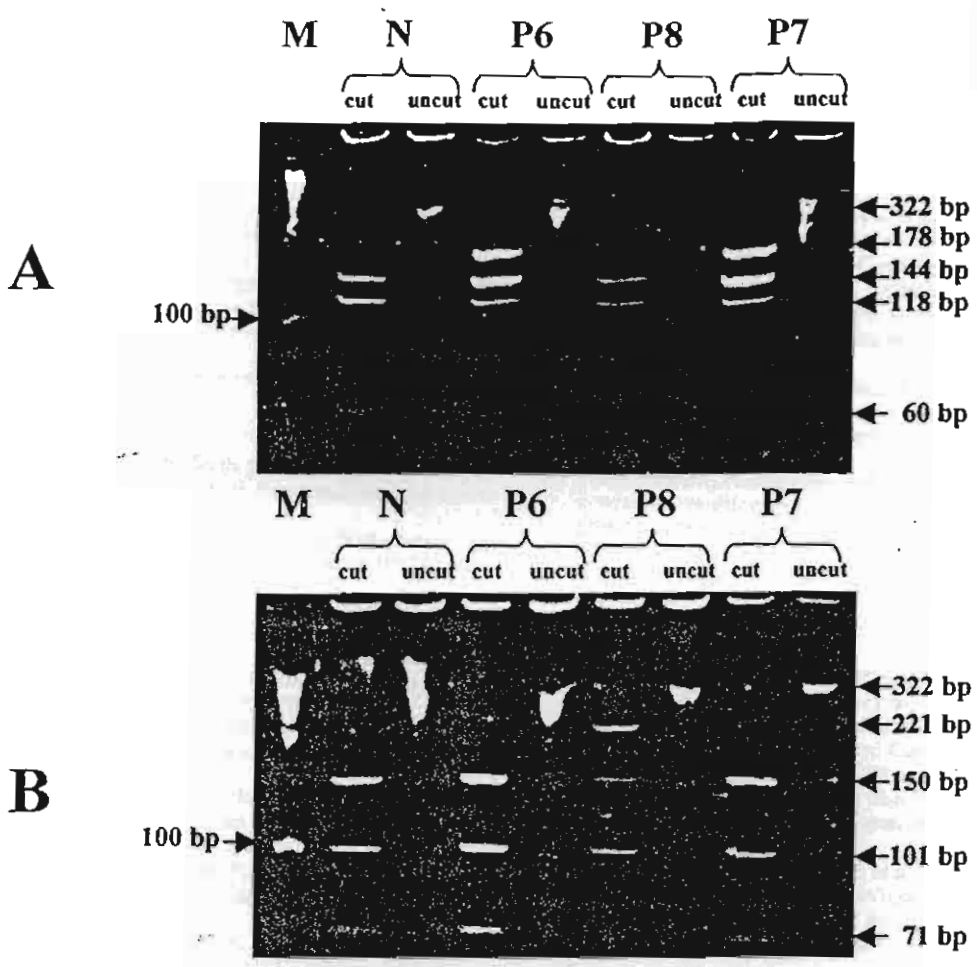

Fig 1 Two mutations causing Apert syndrome arising in *FGFR2* exon 8 are detected by restriction enzyme analysis and electrophoresed on 12% polyacrylamide gel stained with ethidium bromide. (A) The detection of S252W by *Mbo*I, in which individuals with this mutation (P6 and P7) show the 178 bp fragment in addition to the 144, 118, and 60 bp fragments seen in normal individuals. (B) The detection of P253L by *Bgl*I. The presence of the new band of 221 bp in addition to the 150, 101, and 70 bp fragments indicates that P8 is heterozygous for the P253L. In both (A) and (B), M in lane 1 represents a 100 bp marker with the band 100 bp indicated with an arrow; lanes 2 and 3 are controls (N); lanes 4 and 5 are from patient 6 (P6); lanes 6 and 7 are from patient 8 (P8); lanes 8 and 9 are from patient 7 (P7). Cut, PCR products digested by restriction enzymes; uncut, PCR product without adding restriction enzyme and showing only the undigested 322 bp fragment.

craniosynostosis syndromes.[14,20–22] The C278F mutation found in patient 1 was also associated with various clinical syndromes including Crouzon, Pfeiffer, and Jackson-Weiss syndromes.[23–25] Nonetheless, the S347C mutation found in patient 2 has been associated only with Crouzon syndrome.[6,24,26]

While the spectrum of *FGFR2* mutations causing Crouzon syndrome is wide, those causing Apert syndrome are much more restricted.[27] Four of our patients with Apert syndrome had S252W, while the other three had P253R. These two mutations account for approximately 99% of Apert syndrome mutations in a ratio of about 2:1.[6,28] Four of our patients with Apert syndrome had cleft palates: two with S252W and the other two with P253R. Severity of craniofacial malformations and syndactyly was not distinguishable between the two mutations. Negative correlations between phenotypic features and *FGFR2* mutations was consistent with a published study,[29] although some others did report significant correlations.[30,31]

Conclusion

We identified 11 unrelated Thai patients with Crouzon and Apert syndromes. They all had mutations in the *FGFR2* gene supporting the obser-

vation that mutations in the *FGFR2* are responsible for the phenotypes across different ethnic groups. Moreover, the identified molecular abnormalities could be used in developing diagnostic tools to identify prenatal cases in families at risk.

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บทความที่ 18

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Fibroblast growth factor receptor 3 S249C mutation in virus associated squamous cell carcinomas

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Abstract. An S249C mutation in fibroblast growth factor receptor 3 (*FGFR3*) gene was recently identified in patients with cervical carcinomas (CC). However, its importance in cervical tumorigenesis is still inconclusive. Apart from CC, nasopharyngeal carcinoma (NPC) is the other major virus associated squamous cell carcinoma. We sought to clarify the frequency of the *FGFR3* S249C mutation in 75 primary CC in the Thai population and to determine its prevalence in 69 primary NPC by PCR and restriction enzyme digestion. None of the patients but one NPC showed the enzyme digestion pattern consistent with the mutation. This is the first report demonstrating the role of *FGFR3* in the development of human NPC. This study confirms the low frequency of the *FGFR3* S249C mutation in CC. Nevertheless, the discovery of the mutation, not only in CC as reported by previous studies, but in NPC based on this report, suggests that *FGFR3* may play a significant role in human CC and NPC development.

Introduction

Cervical and nasopharyngeal carcinomas are the two major virus associated squamous cell carcinomas. Cancer of the uterine cervix is one of the most common tumors affecting women worldwide with approximately 470,000 new cases diagnosed annually (1). Virtually all cervical carcinomas examined are positive for human papillomavirus (HPV) (2). Even though HPV is considered an essential cause of cervical cancer, it is certainly insufficient to induce transformation and tumor progression (3). A long latency period between HPV infection and tumor appearance is suggested by the fact that the peak incidence of the disease is observed in females above 40 whereas HPV infection occurs in the 20s. Obviously, one important issue is to identify factors marking the transition of the HPV-containing cells to malignancy.

Recurrent genetic alterations in cervical cancer include losses of heterozygosity of many chromosomal regions, recurrent amplification of a few chromosomal sites, and microsatellite instability (3). Specific genes at these loci, however, still remain to be elucidated. Recently, a mutation, 746C→G (S249C), in a gene encoding fibroblast growth factor receptor 3 (*FGFR3*) was identified in 3 of 12 (25%) cervical carcinomas from the French population (4), making it the most common specific molecular genetic alteration in cervical cancer. However, two more recent articles analyzing a larger number of cervical carcinomas refuted the importance of the *FGFR3* activation in cervical tumorigenesis (5,6).

Nasopharyngeal carcinoma (NPC) is relatively common in South China and Southeast Asia with an incidence of 3 to 10 per 100,000 people/year compared to less than 1 per 100,000 people/year in most parts of the world (7,8). Epstein-Barr virus (EBV) appears to be an important etiological factor for NPC (9,10). Several recurrent genetic alterations in NPC have been identified including losses of heterozygosity of many chromosomal regions, recurrent amplification of a few chromosomal sites, and microsatellite instability (11,12). Because of the similarity between cervical cancer and NPC as to their ubiquity in Thailand, their virus associated tumorigenesis, and their histopathology, we sought to clarify the role of the *FGFR3* S249C mutation, the only mutation identified in the *FGFR3* gene in cervical cancers to date, in a large sample of cervical carcinomas in the Thai population and to determine its role in nasopharyngeal tumorigenesis.

Materials and methods

Having obtained informed consent, slides of paraffin-embedded dissected tissues from 23 cervical carcinoma patients, collected between 1997 and 2000, were washed with xylene solution followed by 100%, 95% and 70%

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Key words: cervical cancer, nasopharyngeal cancer, fibroblast growth factor receptor

ethanol, respectively, resuspended in 1 ml lysis buffer (10% SDS, 0.75 M NaCl, 0.24 M EDTA pH 8.0, and 20 µg/ml proteinase K) and rotated overnight at 55°C. Equal volumes of phenol-chloroform were added to the solution and centrifuged at high speed for 15-20 min. DNA was precipitated with 3 M sodium acetate, washed twice with 100% and 70% ethanol, air dried, and resuspended in a volume of 50-200 µl dH_2O.

Primary tissues were collected from 52 patients with cervical carcinoma and 69 patients with nasopharyngeal carcinoma. The tissues were divided into two pieces, the first part was sent for routine histological examination whereas the second part was incubated in 1.2 ml digestion buffer per 100 mg tissue on a shaker at 50°C overnight. This was followed by phenol-chloroform extraction and ethanol precipitation. The resulting pellets were air dried, and resuspended in dH_2O.

The samples were PCR amplified by one of the following two methods. First, using nested PCR for amplification, the primers FR3F7: 5'-AGT GGC GGT GGT GGT GAG GGA GG-3' and FR3R7: 5'-AAT CCT TCA CGC AAC CCG CAG CCA-3' were used for the first round PCR reaction in total volume of 20 µl comprising 200 ng of each primer, 1.2 mmol/l $MgCl_2$, 200 µmol/l dNTPs, 1 U Taq polymerase with the appropriate reaction buffer supplied by the manufacturer's, and 50 ng of genomic DNA. The reaction consisted of 30 cycles at 94°C for 30 sec, 64°C for 45 sec and 72°C for 1 min. A second nested PCR reaction was generated by using 1 µl of the first PCR product and the same reaction mix as above except for primers INF1: 5'-CTG AGC GTC ATC TGC CCC-3' and INR1: 5'-CGC CTG CAG GAT GGG CCC-3'. The nested reaction comprised 5 cycles at 94°C for 30 sec, 64°C for 45 sec and 72°C for 30 sec. These conditions result in amplification of a 55 bp product containing codon 249 in exon 7 of the *FGFR3* gene.

Secondly, the samples were amplified using the FR3F7 and INR1 primers in the 35-cycle reactions at 94°C for 45 sec, 64°C for 45 sec and 72°C for 1 min, from which an 87-bp PCR product containing codon 249 was obtained. We also designed a mutated positive control primer: 5'-CGC CTG CAG GAT GGG CCG GTG CGG GCA G-3' used as the reverse primer for amplification with either the forward primer INF1 applying the first method or the forward primer FR3F7 applying the second method. The PCR products obtained with the mutated primer are of the same size as with the normal primers but contain a nucleotide change at position 746 (746C→G) of codon 249, which serves as a restriction site for the enzyme, *Fnu4HI*. All PCR products were digested with *Fnu4HI* (New England Biolabs) overnight according to the manufacturer's protocol, electrophoresed through a 12% polyacrylamide gel and stained with ethidium bromide.

Results

Of the 75 cervical carcinoma specimens, 55 (73.3%) were squamous cell and 16 (21.3%) were adenocarcinoma. A histology subtype was unavailable for four (5.3%) cases. As for FIGO staging, one was stage IA, 23 were stage IB, two were stage IIA, 15 were stage IIB, 28 were stage IIIB, one

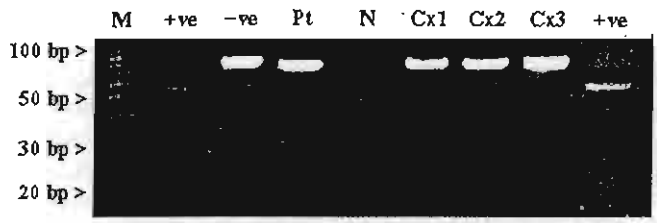

Figure 1. *Fnu4HI* digestion of PCR products. Lane 1 is a 10 bp marker. Lanes 2 and 9 are positive controls containing the *FGFR3* S249C mutation generated by amplification with the mutated reverse primer. Lane 3 is a negative control amplified from white blood cells of a normal person. Lane 4 is the patient #389 demonstrating the 60- and 27-bp bands consistent with the S249C mutant allele. Lane 5 is a control without DNA. Lanes 6-8 are samples obtained from three patients with cervical cancer.

was stage IVA, and two were stage IVB. No stage was available for three cases. None of the 75 samples had a *Fnu4HI* enzyme digestion pattern consistent with the *FGFR3* S249C mutation.

Surprisingly, applying the same method, we found one of the 69 primary nasopharyngeal cancer tissues displaying a pattern consistent with the *FGFR3* S249C mutation (Fig. 1). An 87-bp PCR product of the patient #389 amplified by FR3F7 and INF1 primers was cut into 67 and 20 bp fragments by the *Fnu4HI* restriction enzyme, identical to the pattern of the positive control. The reaction was repeated thrice yielding identical results.

Patient #389 was a 66-year-old Thai male patient. He presented to the hospital because of recurrent epistaxis for 1 year. A right upper jugular lymph node, 2 cm in diameter, was palpated. Sinuscopy revealed ulcers at the roof of the nasopharynx. A nasopharyngeal biopsy was performed and the histopathology revealed carcinomatous changes consistent with undifferentiated type nasopharyngeal cancer. Computerized tomography of the nasopharynx revealed a mass at the nasopharyngeal roof extending to the right parapharyngeal space with right sphenoidal sinusitis. Bone scan showed no metastasis. Pure tone audiogram revealed mild left sensorineural hearing loss and mild right conductive hearing loss. Complete blood counts, BUN, creatinine, plasma glucose, VDRL, erythrocyte sedimentation rate, HIV antibody, prothrombin time, partial thromboplastin time, and chest X-ray were within normal limits. Stage $T_{2B}N_1M_0$ was given. The patient was treated with radical radiation applying 6,700 cGy for 6.5 weeks. At his last visit two years after completion of the radiation treatment, he was still in clinical remission.

Discussion

Fibroblast growth factors constitute a family of related mitogens with at least 18 members characterized to date (13). Four fibroblast growth factor receptors (FGFR1-4) make up a family of structurally related receptors encoded by four different genes (14). These receptors are composed of three extracellular immunoglobulin (Ig)-like domains, a transmembrane domain and a tyrosine kinase domain. The *FGFR3* gene on chromosome 4p16.3 consists of 19 exons spanning 16.5 kb (15,16). Specific point mutations in *FGFR3*

were found associated with several autosomal dominant craniosynostoses and skeletal dysplasias including Muenke craniosynostosis, achondroplasia, hypochondroplasia, and thanatophoric dwarfism (13). Its oncogenic role was first proposed in multiple myeloma (17,18). After the chromosomal translocation t(4;14)(p16.3;q32), the *FGFR3* gene is translocated to the immunoglobulin heavy chain locus at chromosome 14q13. This mutation results in increased levels of *FGFR3* expression, presumably contributing to tumorigenesis of multiple myeloma. In addition, dysregulation of *FGFR3* was recently found in approximately 50% colorectal cancer patients (19). However, there are some human malignancies without evidence of *FGFR3* abnormalities such as human prostate (20) and gastric (21) cancers.

The role of *FGFR3* in tumorigenesis of cervical cancer was first elucidated by Cappellen *et al* (4). After having detected *FGFR3* expression in normal bladder and cervix epithelia, they screened for mutations by PCR-SSCP analysis of the whole coding region of *FGFR3* and found point mutations in 9 of 26 (35%) bladder carcinomas and 3 of 12 (25%) cervical carcinomas from the French population (4). All three cervical cancers had an S249C mutation and were somatic in nature. The identical germ line mutation causes thanotophoric dwarfism, a lethal form of skeletal dysplasia (22).

However, Yee *et al* (5) analyzed 104 primary cervical cancers from the urban Northeastern United States by direct sequencing of the 161-base-pair polymerase chain reaction product containing codon 249 and found only the wild-type sequence. Similarly, Wu *et al* (6) performed sequence-based mutational analysis of *FGFR3* in 51 primary cervical cancers from Columbia, Spain and the Philippines and 7 cervical carcinoma-derived cell lines. They analyzed exons 7, 10, 13, 15 and 19, which encompassed all the previously described *FGFR3* mutations, and found only one primary tumor with the S249C. Collectively, the S249C has been identified in only 4 of 157 primary cervical cancers and none of 7 cell lines. The mutation is somatic in nature.

We analyzed the S249C by PCR-restriction enzyme digestion of 75 primary cervical cancers and found that none of them contained the mutation. This finding reduces the prevalence of the *FGFR3* S249C mutation in primary cervical cancers to 4 in 232. It supports the findings of Yee *et al* (5) and Wu *et al* (6) that the *FGFR3* S249C is unlikely to represent a common mutation in cervical carcinoma. The discrepancy between the data of Cappellen *et al* (4) and our data could be due to different ethnicity or a number of environmental influences such as different HPV strains. Several HPV types have been found associated with this tumor, particularly types 16, 18, 33 and 42 (23). Different types could have different effects. Another possibility would be that there are other mutations affecting this gene in different populations and that with our methods we could only detect the S249C mutation. However, it is the only mutation observed in cervical cancers following a mutation screen of the entire *FGFR3* coding region by Cappellen *et al* (4) and a sequence analysis of all exons encompassing all *FGFR3* mutations previously described by Wu *et al* (6). We believe the results of Cappellen *et al* could have resulted from the small number of samples analyzed.

As there are several similarities between cervical cancers and nasopharyngeal cancers, we analyzed 69 primary nasopharyngeal cancers for the S249C mutation. One of these had the pattern of the *Fnu4HI* enzyme digestion identical to the positive control, which contains the *FGFR3* S249C mutation. Collectively, this patient is the only one out of 144 cervical and nasopharyngeal cancer patients we analyzed who displayed the pattern. As seen in Fig. 1, the undigested 87-bp band of the patient is much denser than the digested 27- and 60-bp bands throughout the three repetitive experiments. We believe this was due to the presence of other normal cells such as lymphocytes, which should contain only normal alleles and hence, not be digested by the enzyme in the specimen we biopsied and used for DNA extraction. Since the S249C is a known mutation and the patient #389 showed the unequivocal, repeatable and identical digestion pattern, we consider sequencing unnecessary. The nature of the mutation should be somatic, not germ line. Unfortunately, the blood sample from the patient is not available to confirm the hypothesis. Nevertheless, this is the first report to demonstrate that *FGFR3* may play a role in the development of human nasopharyngeal carcinoma.

In summary, even though its contribution may be minor, *FGFR3* S249C has been demonstrated to play a role in human cervical and nasopharyngeal oncogenesis.

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บทความที่ 19

ONLINE MUTATION REPORT

Maternal 677CT/1298AC genotype of the *MTHFR* gene as a risk factor for cleft lip

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Non-syndromic cleft lip with or without cleft palate (CL/P) is one of the most common congenital anomalies world wide. It has a prevalence of approximately 1/1000 among white populations[1] and 1/600 among Thai newborns.[2] Environmental and genetic factors have been implicated in CL/P and several different loci and genes have been associated with them.[3]

Maternal folic acid supplementation during early pregnancy may reduce the risk for oral clefts,[4 5] but this is controversial.[6] One of the mechanisms by which low folate levels predispose some subjects to oral clefts could be the presence of polymorphisms in the genes encoding enzymes of the folate pathway, such as 5,10-methylenetetrahydrofolate reductase (*MTHFR*, MIM 236250). MTHFR catalyses the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the predominant circulatory form of folate and the carbon donor for the remethylation of homocysteine to methionine. Two polymorphisms, 677C>T and 1298A>C, in the *MTHFR* gene have been shown to have reduced MTHFR activity.[7 8] The 677C>T transition, producing an alanine to valine amino acid substitution within the catalytic domain of the MTHFR enzyme,[7] has been associated with many disorders and conditions including neural tube defects,[9] vascular disease,[7] migraine,[10] smoking behaviour,[11] and oral clefts.[12 13] However, the last is controversial.[14] Recent studies reported an association between the maternal polymorphism and the anomalies,[15 16] but this again is not a consistent finding.[17 18] No studies have investigated 1298A>C, the second most common polymorphism in *MTHFR* resulting in a glutamate to alanine substitution, in CL/P patients and their parents. We therefore carried out a case-control study to determine whether the two *MTHFR* polymorphisms in Thai patients with CL/P or their parents were associated with an increased risk of the anomaly.

Key points

- Previous data have shown an association between the 677C>T polymorphism in the *MTHFR* gene in either non-syndromic cleft lip with or without cleft palate (CL/P) patients or their mothers and an increased risk of the anomaly, but this finding remains controversial. No studies have investigated 1298A>C, the second most common polymorphism in *MTHFR*, in CL/P patients and their parents.
- We investigated 109 CL/P patients, 67 of their mothers, 45 of their fathers, and 202 controls for the 677C>T and 1298A>C polymorphisms. We found no association between any of the patients' genotypes and CL/P. Transmission disequilibrium test (TDT) analysis also showed no evidence for the association.
- However, a significantly higher frequency of the compound heterozygous 677CT and 1298AC genotype was detected in mothers of CL/P patients with an odds ratios of 4.43 (95% confidence interval 1.33 to 15.10).
- These results indicate an effect of the maternal genotype, rather than in the affected subjects.

SUBJECTS AND METHODS

The study sample consisted of 109 CL/P patients, 67 of their mothers, 45 of their fathers, and 202 controls. Of 109 patients, 14 were familial. All of them were studied under the auspices of the Thai Red Cross, a national charity organisation devoted to providing clinical care for the poor. Subjects were recruited between 2000 and 2002 from seven centres in Thailand (Nakornratchaseema, Nan, Uthaithanee, Maehongsorn, Trang, Srakaew, and Bangkok). As preoperative evaluations, every patient was examined by a geneticist (VS) for any presence of associated anomalies suggestive of syndromic variants. In addition, a family history and epidemiological data were obtained and will be reported elsewhere. After receiving their informed consent, blood samples for DNA analysis were obtained at the time of blood typing and haematocrit determination. Only one patient in families with more than one affected subject was included in the case group. All syndromic cases were excluded. Only the cases of non-syndromic CL/P (normal growth, normal development, and no other major anomalies) were analysed in this report. The control samples were blood donors with no oral clefts in Bangkok and Nakornratchaseema collected in the same period. The study was approved by the institutional review board in Thailand.

DNA was extracted by standard procedures and was amplified using the polymerase chain reaction (PCR). Genotyping for the *MTHFR* 677C>T and 1298A>C polymorphisms was performed by restriction digestion of PCR products with *Hin*fI[7] and *Mbo*II,[19] respectively.

Statistical analysis

Standard chi-square and p values were calculated by a program available at http://quantrm2.psy.ohio-state.edu/kris/chisq/chisq.htm. Haplotype frequencies were estimated by the EH program downloaded from http://linkage.rockefeller.edu/ott/eh.htm. The transmission disequilibrium test (TDT) analysis was carried out on subjects with heterozygous informative parents.[20] Data from families with one parent missing were excluded. Haplotypes of subjects homozygous for at least one polymorphism are readily predicted from their genotypes. All of the 677CT/1298AC genotypes were predicted to be 677C-1298C and 677T-1298A haplotypes because the 677T-1298C haplotype has not been identified.[21] The TDT data were analysed using a $k - 1/k$ correction (where k is the number of alleles).[22] Odds ratios and 95% confidence intervals (95% CI) were calculated from the Epi Info 2000 program, to estimate the relative risk of the different genotype combinations.

Table 1 Frequencies of *MTHFR* alleles in patients with CL/P, their parents, and controls

	MTHFR allele					
	677C*	677T*	χ^2 (p value)	1298A*	1298C*	χ^2 (p value)
CL/P patients (n=109)	0.89 (193)	0.11 (25)	0.11 (0.74)	0.72 (158)	0.28 (60)	0.05 (0.83)
Mothers (n=67)	0.83 (111)	0.17 (23)	1.97 (0.16)	0.69 (93)	0.31 (41)	0.75 (0.39)
Fathers (n=45)	0.87 (78)	0.13 (12)	0.06 (0.81)	0.79 (71)	0.21 (19)	1.22 (0.27)
Controls (n=202)	0.88 (354)	0.12 (50)	–†	0.73 (296)	0.27 (108)	–†

*The number of subjects is indicated in parentheses.
†Reference category.

Table 2 Calculated frequencies of the *MTHFR* haplotypes in patients with CL/P, their parents, and controls from the EH program

Haplotype	677C-1298A	677C-1298C	677T-1298A	677T-1298C	χ^{2*}	p
CL/P patients	0.61	0.28	0.11	0.00	0.14	0.986
Mothers	0.52	0.31	0.17	0.00	3.46	0.494
Fathers	0.66	0.21	0.13	0.00	1.26	0.805
Controls	0.61	0.27	0.12	0.00	–†	–†

*$\chi^2 = 2T$; $T = \{Ln(L)case\} + \{Ln(L)control\} - \{Ln(L)case+control\}$.
†Reference category.

Table 3 TDT analysis in CL/P patients

Haplotype	Transmitted	Untransmitted	χ^{2*}	p
677C-1298A	17	14		
677C-1298C	12	14		
677T-1298A	6	9		
677T-1298C	0	0	0.70	0.811

*χ^2 were analysed using a $k - 1/k$ correction (where k is the number of alleles). Degree of freedom = (number of rows − 1) × (number of columns − 1) = 1 × 2 = 2.

RESULTS

The observed frequencies of the 677C, 677T, 1298A, and 1298C alleles in affected subjects, their mothers, their fathers, and controls are given in table 1. The observed distribution of genotypes among controls was compared with that expected according to Hardy-Weinberg equilibrium: no difference was found (for the 677 locus, $\chi^2=0.314$, 2 df, p=0.855; for the 1298 locus, $\chi^2=0.032$, 2 df, p=0.984). All genotype frequencies of the patients and their parents also followed Hardy-Weinberg equilibrium (data not shown).

The haplotype frequencies of the patients, their parents, and controls calculated from the EH program are shown in table 2. All of the 677CT/1298AC genotypes were expected to be 677C-1298C and 677T-1298A haplotypes. No 677T-1298C haplotype was found. TDT analysis was carried out on subjects with heterozygous informative parents, but it showed no evidence for association of CL/P with any of the haplotypes, as shown in table 3. In addition, the TDT analysis of each individual polymorphism did not show any association between the variant and CL/P (data not shown).

Analyses were carried out to estimate the risk associated with each genotype. The results calculated as odds ratios and 95% CI are reported in table 4 Interestingly, a significantly higher frequency of the 677CT/1298AC genotype was observed in the mothers of CL/P patients compared to controls, with an odds ratio of 4.43, 95% CI 1.33 to 15.10. The odds ratios for any other genotypes in any groups were not increased.

DISCUSSION

We have established *MTHFR* genotypes in 109 CL/P patients, their parents (67 mothers and 45 fathers), and 202 controls. The frequency of the 677T allele in our controls was 12%, which is comparable to the frequencies of 14% in the control population reported in independent studies in Thailand.[23] Our results agree with a previous observation that the polymorphism was found in every population tested and its frequency among Asians is quite similar to that of Europeans, but higher than in Africans.[24] The frequency of the 1298C allele in our controls was 27%, which is similar to that of white populations (27-33%).[8 25]

No 677T-1298C haplotype was detected. This finding agrees with an observation that a subject with the 677TT genotype always had a 1298AA genotype and a subject with the 1298CC genotype always had a 677CC genotype (table 4). In addition, it supports the recent finding that the 677T occurred on a founder haplotype of 1298A.[21]

We found no association between any of the patients' genotypes and CL/P. TDT analysis was carried out on subjects with heterozygous informative parents but also showed no evidence for the association. This finding is consistent with a previous report.[14] However, when we analysed maternal genotypes and CL/P in their offspring, we found the odds ratio calculated for mothers having the 677CT/1298AC genotype, compared to the normal 677CC/1298AA genotype, were 4.43 (95% CI 1.33 to 15.10). There were approximately 12% (eight of 67) of mothers with such a genotype; therefore, folate supplementation in a pregnant woman's diet may benefit these 12% of Thai children who are susceptible to CL/P owing to the 677CT/1298AC genotype in their mother. No such relationship could be found with any genotypes in fathers of affected offspring. This observation is in agreement with two recent independent association studies showing that there is an association between a maternal polymorphism, 677C>T, in the *MTHFR* gene in mothers of affected subjects and an increased risk of CL/P with risk ratios of 2.51 (1.00 to 6.14)[15] and 4.09 (1.32 to 11.57).[16]

A previous study showed that the activity of the MTHFR enzyme in subjects with 677CT/1298AC (47.7%) is higher than that of 677TT/1298AA (24.8%).[8] However, we found that the detected risk of the 677TT/1298AA mothers was no higher than that of the 677CT/1298AC mothers. Although the higher risk is associated with the 677TT/1298AA mothers compared to the normal 677CC/1298AA controls (odds ratio = 3.08), it is not statistically significant (95% CI 0.30 to 31.28). A possible interpretation is that the lower activity of the enzyme increases the susceptibility to fetal loss[26] or to giving birth to children with multiple associated malformations, without

Table 4 Prevalence and calculated odds ratios with 95% CI of the *MTHFR* polymorphisms in patients with CL/P, their parents, and controls

MTHFR 677/1298 genotype	Observed frequency*†				Odds ratio (95% CI)‡		
	CL/P patients (n=109)	Mothers (n=67)	Fathers (n=45)	Controls (n=202)	CL/P patients	Mothers	Fathers
CC/AA	0.30 (33)	0.25 (17)	0.42 (19)	0.33 (66)	0.89 (0.52 to 1.52)	0.70 (0.36 to 1.36)	1.51 (0.74 to 3.06)
CC/AC	0.41 (45)	0.37 (25)	0.31 (14)	0.37 (74)	1.22 (0.73 to 2.01)	1.03 (0.56 to 1.89)	0.78 (0.37 to 1.64)
CC/CC	0.06 (6)	0.06 (4)	0.04 (2)	0.07 (14)	0.78 (0.26 to 2.26)	0.85 (0.23 to 2.91)	0.62 (0.09 to 3.05)
CT/AA	0.20 (22)	0.16 (11)	0.16 (7)	0.20 (40)	1.02 (0.55 to 1.90)	0.80 (0.36 to 1.74)	0.75 (0.28 to 1.91)
CT/AC	0.03 (3)	0.12 (8)	0.02 (1)	0.03 (6)	0.92 (0.18 to 4.26)	4.43 (1.33 to 15.10)	0.74§
CT/CC	NO	NO	NO	NO	ND	ND	ND
TT/AA	NO	0.03 (2)	0.04 (2)	0.01 (2)	ND	3.08 (0.30 to 31.28)	4.65 (0.45 to 47.82)
TT/AC	NO	NO	NO	NO	ND	ND	ND
TT/CC	NO	NO	NO	NO	ND	ND	ND

*The number of subjects is indicated in parentheses. †NO = not observed. ‡ND = not determined. §The 95% CI is not determined owing to the invalidity of the Cornfield 95% confidence limits for odds ratio.

increasing the risk of CL/P alone. Another explanation would be a limited number of mothers with the 677TT/1298AA genotype in our study owing to its low frequency. Although not statistically significant, a higher risk is associated with the 677TT/1298AA fathers compared to the normal 677CC/1298AA controls (odds ratio = 4.65), which is also greater than that observed for the 677TT/1298AA mothers. Further studies with more numbers of fathers are needed to determine the association.

Three previous studies, however, showed no association between the maternal 677TT genotype and CL/P.[17 18 27] Our results do not contradict these reports because the three studies did not determine the genotype at the 1298 position. Moreover, the inconsistent result could be caused by the difference in populations studied with diverse genetic backgrounds and environmental factors. In addition, the timing of sample collections, whether it was before or after folic acid fortification of foods, started in the USA in 1998, could affect the frequencies of the polymorphisms in subjects with some anomalies.[28]

These findings indicate a possible involvement of the folate pathway in the causation of CL/P, and support an influence of the maternal genotype, rather than an effect of the embryo's genotype. The 677C>T polymorphism converts an alanine to a valine residue at position 222 making the MTHFR thermolabile with reduced activity to 65% in heterozygotes and 30% in homozygotes.[7] The 1298A>C changes a glutamate into an alanine residue and reduces the enzyme activity to 83% in heterozygotes and 61% in homozygotes. Although neither the homozygous nor the heterozygous state for the 1298A>C is associated with raised plasma homocysteine or decreased plasma folate levels, the combined heterozygosity for 677C>T and 1298A>C is associated with higher homocysteine and lower plasma folate concentrations.[8] Nonetheless, the real detailed biological mechanism remains to be elucidated.

So far, this is the first report on the investigations of both 677C>T and 1298A>C polymorphisms in the *MTHFR* gene of patients with CL/P and their parents. Our results indicate an effect of the maternal genotype, rather than that of the affected subjects.

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